APPLICATIONS OF A RADIOIMMUNOASSAY TECHNIQUE TO THE STUDY OF LUTEINIZING HORMONE SECRETION IN THE RAT

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MASTER OF SCIENCE

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A B S T R A C T

A sensitive and reproducible double antibody radioimmunoassay technique, requiring 50µl of unknown serum or plasma per assay tube, is described for use with ^{125}I and rabbit anti-rat LH serum.

The assay system was applied to the study of LH secretion in rats under both normal and experimentally manipulated conditions. Particular attention was focussed upon comparison of circulating LH levels in conscious, unstressed animals with those in anaesthetized animals, with or without surgical stress. Thereafter, the effects of acoustic stimulation and of exogenous LRH administration were studied in conscious and anaesthetized animals. Urethane anaesthesia exerted a profound effect upon the LH-secretory response to exogenous LRH in male rats.

Available evidence suggests that the blood sampling method, surgical stress and anaesthesia are each capable of significantly influencing LH secretion, thereby emphasizing the value of studies using conscious, unstressed animals. While a direct effect of urethane on the pituitary gland cannot be excluded, attention is drawn to the possible mediation of a urethane-sensitive inhibitory influence in the mechanism controlling LH secretion in the rat.

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I N T R O D U C T I O N

The present study was embarked upon with the prime objective of establishing a sensitive and reproducible rat LH radioimmunoassay technique requiring minimal volumes of unknown serum or plasma. Thereafter, the assay was used to accumulate baseline data in an inbred strain of Wistar rats used in the Medical School for the last 20 years. Since anaesthesia might interfere with the normal hypothalamic-hypophysial control of gonadotrophin secretion, measurement of serum LH levels in conscious, unstressed rats was considered to be an important advance over published data. This was performed by removal of blood through a chronically placed jugular cannula (203). Plasma LH levels in conscious and anaesthetized rats were thus compared.

The histological findings of Zondek and Tamari (229) have suggested the occurrence of facilitated gonadotrophin secretion, following audiostimulation, in both adult female rats and rabbits. Since plasma LH concentrations have never been estimated in rats following an audiostimulation program, experiments were designed to measure circulating LH levels before and during various sound treatment sessions.

A commonly used anaesthetic in neuroendocrine investigations is urethane. The little information available on the effects of this drug on gonadotrophin secretion is controversial. It was therefore appropriate to measure the effects of urethane on LH secretion in the rat. These were assessed by comparing hormone levels in conscious and urethane-anaesthetized rats subjected to various treatments - i.e. bilateral ovariectomy, surgical stress and intravenous LRH administration.

C H A P T E R I

CONTROL OF ADENOHYPOPHYSIAL GONADOTROPHIN SECRETION

CONTROL OF ADENOHYPOPHYSIAL GONADOTROPHIN SECRETION

Probably the earliest proof of the existence of an internal secretion ("hormone") was presented by Berthold (21) in the 19th century, who transplanted testes into castrated cocks and observed that these transplants were capable of maintaining the normal male development of the birds. A similar study by Knauer (121) 50 years later, involving the grafting of pieces of ovaries into ovariectomized guinea-pigs, showed that this procedure prevented the occurrence of castration atrophy of the accessory reproductive organs. This was soon followed by the work of Marshall and Jolly (132), who made a detailed study of such transplants and published illustrations of microscopic sections of transplanted ovaries. Ovarian tissue, successfully transplanted, behaved in an apparently normal way. This included follicular ripening, ovulation and formation of corpora lutea.

It has long been known that the ovary of an immature animal, when grafted into an adult host, undergoes rapid development and assumes adult function long before it would normally have done so (98). Conversely, ovaries from adult animals, when grafted into immature hosts, lose their functional activity and become quiescent. Goodman (82) and Deanesly (44) demonstrated that ovaries transplanted into castrate male hosts became active, as shown by development of ripe follicles, but that ovulation and formation of corpora lutea did not occur unless the animal received an injection of pituitary extract.

More recent findings suggest that these original studies are explicable in terms of a complex series of feedback effects involving secretion of hypothalamic neurohormones, gonadotrophins

of the anterior pituitary gland, together with the direct and indirect modulating effects of the gonadal steroids on the "higher centres" of control.

The following review of the literature is intended to bridge the gap between the early, pioneering studies and modern concepts of the control of adenohypophysial gonadotrophin secretion.

EVIDENCE FOR CENTRAL NEURAL CONTROL OF REPRODUCTIVE FUNCTION.

Since the time of Aristotle it has been appreciated that environmental factors are capable of influencing reproductive function in animals (98). While it is now common knowledge that menstrual disturbances are often associated with emotional crises, numerous other "external" factors are equally capable of influencing gonadal function. Many external factors exert their influence on the reproductive system via central nervous pathways. The effects of nutrition, temperature, light, sound and emotional factors have been studied in some detail:

Nutrition: Mulinos and Pomerantz (150) have observed that malnourished rats develop a condition similar to that following hypophysectomy, an effect which may be compensated by exposing the animals to additional illumination (1).
The findings of Werner (218) indicate that the ovarian atrophy consequent to starvation may be prevented by the administration of either anterior pituitary extract or pregnant mare's serum, the latter finding strongly suggesting a link between food deprivation and gonadotrophic activity of the anterior pituitary gland. Further studies by Howland (108) suggest that the decreased gonadotrophin secretion following malnutrition may be

due to a decrease in hypothalamic secretion of luteinizing hormone-releasing hormone (LRH). It should be noted, however, that in these studies it is extremely difficult to distinguish between the stress effects of malnutrition and the malnutrition per se.

Temperature: The female rat has been found to exhibit lengthened oestrous cycles when maintained at a low ambient temperature (30, 125) and repeated exposure of young rats to cold significantly advances the time of vaginal opening (130). Furthermore, Wells and Zalesky (217) have demonstrated that the limited period of testicular activity in the male ground squirrel can be greatly extended by keeping the animals in a constant low temperature environment.

Light: There is abundant evidence to suggest that light and sound (page32) play an important role in affecting gonadotrophin secretion. Rowan (181) was one of the earliest workers to demonstrate that extra illumination during the hours of darkness would result in active gonads in the migratory junco finch. Similar findings have been reported by Baker and Ranson (10) with the field mouse, and by Bissonette (25) with ferrets. The fact that hypophysectomized ferrets do not respond to extra illumination (105) suggests that the effect of light on the reproductive system is mediated via the anterior pituitary gland. Studies involving interruption of optic pathways are in agreement with this hypothesis (26, 39).

Constant illumination, in addition to inducing persistent oestrus and premature puberty in the rat, also markedly enlarges the neurones of the supraoptic nucleus (65). Since this region of the brain is known to be associated with secretion of anti-diuretic hormone (ADH), it is interesting to note that

Hagino (91), who exposed baboons to constant illumination, showed that the animals developed polydipsia and polyuria, although menstrual cycles and ovulation remained unaffected. However, adult cycling female rats, when exposed to the same conditions as the baboons, developed persistent vaginal cornification and follicular ovaries devoid of corpora lutea. Recent studies by Piacsek and Hautzinger (167) have demonstrated accelerated sexual maturation of female rats following continuous light exposure from the time of birth. Furthermore, the authors have reported an apparent correlation between light intensity (under conditions of cyclic exposure of 14 hours/day) and the time of onset of puberty, with increase in light intensity evoking increased ovarian and uterine weights, thus suggesting elevated gonadotrophin secretion. In contrast to these findings, which apply to the common laboratory animals housed under controlled, though artificial, environmental conditions, it is well established that sheep may be brought into oestrus if subjected to gradually decreasing duration of light each day (226).

The Pineal Gland: At this juncture it seems appropriate to consider the pineal gland since this endocrine organ, which possesses connections with the visual system, is believed to exert significant influences on gonadal function. Pinealectomy is reported to advance the time of puberty if the operation is appropriately timed during infancy (119), and to induce hypertrophy of the reproductive organs in adult animals (177). It is now generally accepted that the pineal gland elaborates a substance or substances which exert anti-gonadotrophic actions on the ovaries of intact (222) and unilaterally ovariectomized (214) rodents. The suppressive influence exerted by the pineal gland on the pituitary-gonadal

axis in adult male rats has been demonstrated in experiments by Motta et al (149) in which the weights of the seminal vesicles and prostate, 12 days after pinealectomy, increased by 83% and 48% respectively. Pineal extracts, however, delay puberty, induce some gonadal atrophy and interrupt constant oestrus in rats (119, 141). According to Wurtman, Axelrod and Chu (223), many of these effects may be induced by melatonin, which they consider to be a true hormone formed specifically by the pineal body. Furthermore, Motta et al (149) have shown that administration of melatonin to prepuberal female rats delays puberty and results in significant decreases in pituitary, ovarian and uterine weights. Fiske and co-workers found that exposure of rats to continuous illumination (66) for 9 to 10 weeks beginning in infancy, significantly reduced pineal weight, an effect not influenced by removal of the adrenals or gonads. Recent work by Reiter and Johnson (178) with hamsters indicates that bilateral orbital enucleation, or surgical removal of the inner retinal layers, leads to testicular atrophy associated with decreased prolactin and luteinizing hormone (LH) levels in the anterior pituitary. This effect was abolished by either pinealectomy or superior cervical ganglionectomy. The authors suggest that the pineal gland, in light-deprived hamsters, restricts either the synthesis or release of luteinizing hormone-releasing factor (LRH) and prolactin-inhibiting factor (PIF) within the hypothalamus. The evidence presented suggests that the pineal gland tends to exert an inhibitory effect upon gonadal function. Both continuous light exposure and pinealectomy increase the frequency of oestrous vaginal smears, though the magnitude of the response is considerably greater after exposure of rats to continuous artificial illumination. In the latter situation, the incidence of oestrous smears may

reach 100% (177). Thus, while the effects of the pineal gland on gonadal function are somewhat similar to the effects of light per se on reproductive function, all the reported effects of light exposure need not necessarily be mediated by the pineal system.

Reflex Ovulation: In most birds the occurrence of ovulation in the breeding season is dependent upon some stimulus received from the male. Matthews (133) found that the isolated female pigeon did not ovulate, though if the bird was confined with, or in view of a male, another female or even a mirror, then ovulation and oviposition would occur. The rabbit is a reflex ovulator and requires some stimulus, normally supplied by coitus, for ovulation to occur. Haighton (92), in 1797, reported to the Royal Society of London that copulation in the rabbit was normally followed by rupture of the Graafian follicles, but the term "reflex ovulation" was later coined by Heape (102). Reflex ovulation has also been reported in the cat (201).

Emotional Factors: Theobald (212) observed that the menstrual cycle may be influenced by hypnosis as well as by a variety of psychological disturbances. Bass (12) reported the occurrence of amenorrhoea in 54% of women in concentration camps, but noted that the loss of cycles occurred long before other effects of malnutrition were manifest. Similar observations were made by Stroink (205) who attributed a psychic origin to the menstrual disturbance. The possible influence, however, of early malnutrition in such studies on the hypothalamo-hypophysial system cannot be ignored.

Amenorrhoea is also a necessary criterion for the diagnosis of the psychological feeding disorder anorexia

nervosa (98). Following the use of both bioassay (182) and radioimmunoassay (24, 15) techniques, this condition is now known to be associated with altered patterns of gonadotrophin secretion. In addition to the possible effects of malnutrition per se, it has been suggested that emotional factors, and possibly hypothalamic dysfunction, may account for the observed endocrine changes in these patients.

Other examples of failure of higher centre control of reproductive function include the occurrence of amenorrhoea produced by fear of pregnancy in unmarried girls (72), and with the belief that excessive desire for a child may contribute to sterility (13).

The most likely hypothesis to account for the fact that exteroceptive and psychological factors modify the breeding, oestrous and menstrual cycles of so many animal species is that the central nervous system controls the secretion of gonadotrophic hormone from the pituitary gland. The alternative hypothesis that the nervous system regulates the gonads by a secreto-motor nerve supply finds little support since
a) most workers believe that most nerve fibres to the gonads accompany and innervate the blood vessels (98); and
b) exteroceptive stimuli, such as coitus in the rabbit, can still produce ovarian responses in the transplanted organ (71).

ANATOMY OF THE HYPOPHYSIAL PORTAL BLOOD VESSELS

Rioch, Wislocki and O'Leary (179) have defined the main regions of the pituitary gland as the neurohypophysis and adenohypophysis.

The neurohypophysis, derived embryologically from an evagination of the floor of the third ventricle, consists of three parts - the median eminence, infundibular stem and infundibular process, or neural lobe. The first two parts being collectively referred to as the infundibulum or neural stalk. The adenohypophysis, derived embryologically from Rathke's pouch (an evagination of the roof of the pharynx), is also divided into three parts - the pars tuberalis, pars intermedia and pars distalis. The neural stalk, together with its sheath of adenohypophysial tissue, is referred to as the hypophysial stalk. Of particular interest in this discussion is the system of "hypophysial portal blood vessels" which constitutes a direct vascular link between the neural tissue of the median eminence of the hypothalamus,and the tissues of the anterior pituitary gland.

Hypophysial Portal Vessels: Small arterial twigs from the internal carotid arteries and circle of Willis run to supply a rich vascular plexus situated in the pars tuberalis. From this plexus there arises a multitude of capillary loops which penetrate into the tissue of the median eminence and there come into intimate relationship with nerve fibre tracts running through the hypothalamus. These capillary loops are referred to as the primary plexus of the hypophysial portal vessels, and the blood from this plexus is drained down the large portal trunks which lie mainly on the anterior and ventral surface of the pituitary stalk. The portal trunks then break up into a second system of

capillaries (hence their designation "portal") and distribute their blood into the sinusoids of the pars distalis of the adenohypophysis. While the exact pattern of the portal vessels varies from species to species, in each case the close relationship between the primary capillary loops and nerve fibres in the median eminence is strikingly apparent (98).

Direction of blood flow: Following the pioneering work of Popa and Fielding (168,169), the basic anatomical features of the portal vessels was established and later confirmed by Wislocki and King (221) and Wislocki (220). The direction of blood flow through these vessels was believed at that time to be toward the median eminence from the hypophysis. Wislocki, on histological evidence, favoured the view that the blood flowed towards the pituitary gland, with the lower end of the portal vessels extending into the tissue of the pars distalis.

As a result of considerable controversy, Green and Harris (85) made a thorough re-investigation of the portal vascular system. This provided information which enabled them to state with certainty that the direction of blood flow was from the median eminence towards the pituitary. These studies involved histological investigations, both before and after perfusion of the vascular system with indian ink, as well as direct observation of blood flow through these vessels in the anaesthetized animal. Clearly, the latter type of study provides the most conclusive information.

The most likely hypothesis to account for the unique structural characteristics of these vessels is that the nerve fibres of the hypothalamus liberate some humoral substance(s) into the capillaries of the primary plexus, and that this substance is carried by the portal vessels either to promote

or to inhibit secretion by the gland cells in the pars distalis of the adenohypophysis (98).

Regenerative capacity of portal vessels: The hypophysial portal blood vessels have been shown to possess remarkable powers of regeneration following section of the pituitary stalk. Following this operation, Harris (97) demonstrated that capillary outgrowths bridge the gap of the divided stalk as early as 24 to 48 hours later, and that the relatively large vascular trunks may cross the site of section within a few weeks. Furthermore, the capillary network is capable of penetrating the interstices of a plug of cotton wool, gun-cotton or even waxed paper placed between the cut ends of the stalk.

HYPOTHALAMO - ADENOHYPOPHYSIAL RELATIONSHIP IN THE CONTROL OF REPRODUCTIVE FUNCTION

One of the earliest attempts to transplant pituitary tissue was made by Crowe, Cushing and Homans (42) who made transplants into the rectus muscle, bone marrow and cerebral cortex of dogs. Many workers have since transplanted anterior pituitary tissue to distant sites in the body including the anterior chamber of the eye, the testicle and thigh muscles, and have also studied transplants of cultured pituitary tissue (see Harris (98) for references). The results of these diverse studies suggest that there is little evidence in favour of the anterior pituitary gland being able to maintain normal functions if transplanted to a distant site in the body, and that in this respect the anterior pituitary differs from the gonads discussed earlier.

In an original study Greep (87) reported that 28-day-old hypophysectomized rats, bearing auto- and homografts of pituitary tissue in the sella turcica, showed good anterior pituitary function as reflected by growth, oestrous cycles, pregnancy and lactation. Evidence accumulated, following Greep's observations, that the functional activity of the anterior pituitary gland is dependent upon its blood supply via the hypophysial portal blood vessels. It appeared that Greep's findings were a consequence of regeneration of these vessels between the hypothalamus and the grafted pituitary tissue. In order to test this hypothesis, Harris and Jacobsohn (100, 101) carried out a classic study. Adult female hypophysectomized rats were grafted with pituitary tissue taken from their own new-born young, the tissue being inserted into either the subarachnoid space under the cut pituitary stalk,

or laterally under the temporal lobe of the brain. All 12 animals with grafts placed under the pituitary stalk regained a normal oestrous rhythm. On placing them with normal males, half became pregnant and delivered living young, though the milk-ejection reflex was interrupted through lack of functioning posterior pituitary tissue. Post-mortem examination of the animals revealed ovaries, reproductive tracts, adrenals and thyroids that were indistinguishable from those of the normal control animals. Serial sections through the grafts and surrounding structures demonstrated that the grafted tissue had acquired rich vascular connections with the portal vessels, and that the anterior lobes of the grafts contained well differentiated cells of all types, though the neural lobes were atrophic. Those rats in which pituitary tissue was placed under the temporal lobe of the brain showed no oestrous cycles, and post-mortem examination revealed the ovaries, reproductive tract and thyroids to be as atrophic as those of the hypophysectomized control animals. Microscopic examination of the temporal lobe grafts demonstrated the survival of as much pituitary tissue, which was as well vascularized, as in the case of the pituitary stalk grafts.

In a further study (98) the anterior pituitary tissue of adult male rats, placed under the pituitary stalk of hypophysectomized females, supported normal oestrous cycles and pregnancy, indicating gonadotrophin secretion after the female pattern.

These studies demonstrated several important points:

a) The posterior pituitary gland plays a role in the process of milk-ejection.

b) The pituitary gland functions normally only in the

presence of an intact and patent system of hypothalamo-hypophysial portal blood vessels.

c) Anterior pituitary tissue does not show sexual differentiation.

d) Maturation of anterior pituitary tissue.

Post-parturient rats in which grafts of pituitary tissue from their own young were placed under the pituitary stalk, showed the recurrence of normal, regular oestrous cycles at a time when their litters would have been only 12 to 43 days old. This demonstrated that immature pituitary tissue grafted into adult hosts showed hastened development and, together with the fact that immature ovaries grafted into adult hosts also showed hastened development, indicated that the onset of pituitary gonadotrophin-ovarian activity at puberty depends upon some factor, other than maturation of pituitary and ovarian tissues, which probably emanates from the hypothalamus (98).

HYPOTHALAMIC CONTROL OF ADENOHYPOPHYSIAL FUNCTION

Despite speculation nearly 40 years ago that neuro-hormones (or releasing hormones) might exist (99), it is only recently that some of these factors have been isolated, characterized and synthesized (192, 189). The process of neuro-secretion, already shown to occur in the neurohypophysis (63), has not been conclusively demonstrated, at the present time, to be the mechanism of control of adenohypophysial secretion. However, current theories of hypothalamic regulation of anterior pituitary function are based largely upon several observations:

a) Although the anterior pituitary gland receives no direct innervation (84), the existence of a system of hypothalamo-hypophysial portal blood vessels, carrying blood to the pituitary gland, has been confirmed in many species (43).

b) Normal pituitary function depends not only upon a normal hypothalamus and pituitary, but also upon an intact and patent system of portal vessels. This is supported by numerous experiments in which the anterior pituitary gland has been deprived of its blood supply.

c) Extracts of hypothalamic tissue have been found to stimulate secretion of hypophysial hormones. The active components have been purified, characterized, chemically synthesized (192, 189) and biosynthesized (145) in vitro. Direct action of these extracts on the pituitary has been demonstrated in vitro using both crude and purified preparations (89), and in vivo by perfusion of the pituitary either by injection of extracts into the portal vessels (113), or by infusions through intracranial cannulae. In addition, a dose-response relationship has been demonstrated in sheep and rats (78) using homologous hypothalamic extracts, and in man using porcine LRH (114). Furthermore, the synthetic gonadotrophin-releasing hormone has been shown to stimulate the release of both LH and FSH in human subjects (23). Schally and Bowers (190), using chronically ovariectomized, oestrogen-progesterone treated rats, have also shown a close similarity between the in vivo and in vitro pituitary response to LRH of bovine and ovine origin. Studies by Ondo and co-workers (161) demonstrated that injection of synthetic LRH into the third ventricle of rats gave rise to an elevation of plasma LH concentration within 10 minutes, suggesting rapid transport of LRH from cerebrospinal fluid (CSF) to the portal vessels. Similar injections into the cisterna

magna evoked smaller increases in plasma LH concentration, and only after a considerably longer latent period.
Furthermore, it is suggested that hypophysiotrophic substances may be elaborated at sites remote from the median eminence and reach the latter through bulk flow of CSF (161). Ben-Jonathan et al (18) studied the capacity of the median eminence to transport LRH from CSF to hypophysial portal blood, and detected LRH in portal blood as early as 15 minutes after intraventricular injection of 125ng. Intravenous injection of LRH stimulated LH release faster than did intraventricular injection, but the duration of action was shorter via the intravenous route.

While systemic injection of physiological doses of releasing hormones exert a negligible effect on adenohypophysial secretion (189, 78), direct injection of a comparable dose into the portal vessels is highly active in stimulating secretion of pituitary hormones (113).
Furthermore, evidence suggests that doses of LRH, greatly in excess of the physiological, are capable of stimulating the secretion of LH and FSH following either intranasal (129), intravenous, intramuscular or subcutaneous administration (148).

Recent studies by Vilchez-Martinez et al (215), using an antagonist to LRH (des-His^{2}-des Gly^{10}-LH-RH ethylamide) have demonstrated that intravenous infusion of the antagonist for 2 hours significantly inhibits LH release in ovariectomized oestrogen-progesterone treated rats, as well as in adult males. Furthermore, in cycling female rats, 2 injections of antagonist effectively blocked ovulation in response to exogenous LRH.

These findings all confirm the importance of the portal blood vessels, which permit nanogram and picogram quantities

of hypothalamic factors to gain access to the pituitary gland in relatively high concentration. The subsequent dilution in the body fluids, together with rapid degradation in the plasma (176), would render recirculation effects minimal.

The above observations confirm that secretion of anterior pituitary hormones occurs in response to secretion of hypothalamic releasing or inhibiting hormones respectively, secreted directly into the portal capillaries from the hypothalamic nerve endings and reaching the pituitary gland via the portal blood supply. Specificity of the neurohormones is relative, rather than absolute, as shown by the secretion of both FSH and LH in response to LRH (189). It has been proposed that a single molecule might be responsible for the release of both FSH and LH (219). An alternative theory proposes "tonic" secretion of hypothalamic releasing factors (3) coupled with changes in sensitivity of the anterior pituitary gland to the influence of these different factors at different stages of the reproductive cycle - e.g. the preovulatory surge of LH at proestrus would reflect maximum sensitivity of the anterior pituitary gland to LRH (3). The regulation of pituitary function may therefore involve a complex interaction between changing concentrations of neurohormones in portal blood, coupled with changing pituitary sensitivity to these releasing hormones, thus accounting for the ability of LRH to increase serum FSH levels (189, 191). Since serum FSH and LH do not fluctuate in parallel in the reproductive cycle in man (111) and in rats (77), it is thought that the differential release of LH and FSH from the pituitary gland could perhaps be modulated by sex steroids (191). Indeed, steroids are thought to play an important role in altering pituitary sensitivity to the releasing factors as implantation of steroids into the hypothalamus influences secretion of

gonadotrophins (68). It is possible that a single LH/FSH releaser molecule is modified by pituitary enzymes to endow it with appropriate activity (191). Studies by Franchimont et al (69) suggest that the secretory responses of the gonadotrophins to LRH are influenced by the endocrine equilibrium, and more particularly by the interaction of the gonadal steroids which can alter the release and/or synthesis of the pituitary gonadotrophins.

In addition, periodic surges of LH release have been demonstrated in castrated monkeys (47), rats (80) and sheep (75) and in intact men (153), women (142) and sheep (157). The surges occur at regular time intervals in a given species, with maximal secretion occurring at 30 minute to 4 hour intervals. Studies by Blake and Sawyer (29), involving varying degrees of deafferentation of the medial basal hypothalamus (MBH), provide evidence that both the post-castration rise in plasma LH concentration and the pulsatile nature of LH secretion might be autonomously inherent to the medial basal hypothalamo-hypophysial unit. Thus, the pulsatile nature of LH release may be accounted for in terms of changes in hypothalamic neuronal activity, rather than by changes in pituitary sensitivity to LRH.

NORMAL PATTERNS OF GONADOTROPHIN SECRETION

Ramirez and McCann (174), using the ovarian ascorbic acid depletion assay of Parlow (165), demonstrated that in cyclic female rats, LH was detectable in the plasma in significantly large amounts on the afternoon of proestrous, but not at other times during the reproductive cycle - a finding confirmed by Anderson and McShan (2). Numerous workers, using radio-

immunoassay techniques, have studied circulating levels of both LH (147, 158, 195, 151) and FSH (195) during the normal oestrous cycle of the rat, and have found marked rises in the concentrations of both hormones on the afternoon of proestrous. According to Monroe et al (147), the highest incidence of elevated circulating LH levels occurred between 3 and 6pm, with mean levels associated with ovulation of the order of 400 to 800 ng/ml.

Furthermore, estimations of pituitary LH and FSH contents at various stages during the cycle (195, 151) have revealed that a sudden decrease in pituitary LH content occurs at the time of ovulation, and this is associated with a corresponding elevation of serum LH level. Thereafter, pituitary content of gonadotrophins gradually increases to reach peak levels prior to the next ovulation. Since hypophysial LH content is lower on the morning after ovulation than at any other time in the oestrous cycle (193), it appears that the quantity of LH released during the ovulatory surge is well in excess of the minimum ovulation quota.

The question as to whether FSH forms part of the ovulation-inducing complex is uncertain. However, the observation that pituitary FSH content is lowest just prior to ovulation (31), is in keeping with the reported proestrous FSH surge which is coincidental with the LH surge (77).

CONTROL OF GONADOTROPHIN SECRETION

The pioneering work of Fischera (64) demonstrated that removal of the gonads in several different species and in both sexes was followed by enlargement of the pituitary gland with formation of "castration cells". Carmichael and Marshall (33) subsequently reported that, if one ovary is removed from the

rabbit, the other may double in weight. This has been called "compensatory ovarian hypertrophy".

The well documented increased release and synthesis of LH in the gonadectomized rat (88) is consistent with a negative feedback of gonadal steroids which acts to inhibit release of the trophin hormone in the normal animal. Evidence for this view has been presented by McCann and Ramirez (136), who studied the effects of oestrogen or progesterone pretreatment of ovariectomized rats on LH secretion. The results of these studies suggest that both oestrogen and progesterone are capable of inhibiting LH release, but that to be effective in physiological doses, progesterone must act in the presence of oestrogen.

INFLUENCE OF GONADAL STEROIDS ON THE HYPOTHALAMO-PITUITARY AXIS

It is now well known that administration of excessive amounts of oestrogens to normal rats results in gonadal atrophy. After daily treatment for two months with 25 to 50ug of oestradiol, the ovaries contain neither growing follicles nor corpora lutea (67) an effect which has been confirmed in different animal species and in women (88). In experiments on rats, 2ug of oestradiol injected daily decreases serum FSH significantly in castrated male and female animals (74). Studies in ovariectomized animals with rostral hypothalamic cuts have provided good evidence that the negative feedback action of oestrogens on gonadotrophin secretion occurs in the medial basal hypothalamus (MBH). The usual post-castration rise in LH was not altered by the lesions, suggesting that a negative feedback of oestrogen involves regions caudal to the lesion, namely in the hypothalamus (29,207). Furthermore, the

negative feedback arrangements required for compensatory ovarian hypertrophy involve neurones whose connections are lost by anterior cuts in the hypothalamus at the rostral border of the ventromedial nuclei (VMN) and arcuate nuclei (123).

Animals with lesions which disconnect the medial preoptic area (MPOA) from the medial basal hypothalamus (MBH) stop cycling and fail to ovulate (207) but are still capable of compensatory ovarian hypertrophy (123). Since these animals are found to be in constant oestrus their ovaries presumably can maintain oestrogen secretion. This finding supports the view that a continuous release of gonadotrophins can be sustained but oestrous cycles cannot be maintained (136) in animals with anteriorly deafferented MBH (207).

CONTROL OF "TONIC" LH SECRETION

The time course of circulating LH during the menstrual cycle of the Rhesus monkey (146), like that of man, is characterized by low, relatively constant levels interrupted once every 28 days by abrupt elevations in the concentration of this hormone which last 2 to 3 days. These circulating levels of LH in primates may be thought of as effects of tonic secretion and intermittent, or cyclic, discharges of this hormone, the latter inducing ovulation some 24 to 36 hours later.

Studies by Atkinson *et al* (9) with Rhesus monkeys have shown that interruption of the negative feedback loop by gonadectomy leads to elevated plasma LH levels, the latter approaching 10 times basal level in 20 days. In chronically ovariectomized female monkeys, elevated plasma LH concentration represents the integration of pulsatile discharges of

pituitary LH, with a mean frequency of one LH "burst" per hour - hence the appelation "circhoral" rhythm (47). These pulsatile discharges are interrupted by a single intravenous injection of oestradiol, the inhibition lasting long after the circulating oestrogen levels have returned to very low levels. The duration of inhibition is related to the magnitude of the oestrogen pulse. It is interesting to note that this acute closure of the negative feedback loop by injected oestrogen is mimicked by single intravenous doses of haloperidol (an anti-dopaminergic drug) and of the alpha-adrenergic blocking agents phenoxybenzamine or phentolamine (48). In the light of these studies it appears that the circhoral pulses of LH secretion result from signals from the central nervous system, relayed to the pituitary gland by LRH. Furthermore, it has also been found that acute administration of progesterone alone to ovariectomized monkeys (such that plasma concentration is 1,000 times greater than in the luteal phase of the cycle) does not influence the open loop pattern of LH secretion, as does chronic progesterone treatment. The suggestion is made that tonic LH secretion, as reflected in the circulating levels of this hormone during the follicular phase of the cycle, can be accounted for in terms of the negative feedback action of oestrogens alone, and that this negative feedback loop may be influenced by both anti-dopaminergic and anti-adrenergic agents (48), thus implicating dopaminergic and/or adrenergic components respectively (122).

POSITIVE FEEDBACK CONTROL OF PREOVULATORY LH "SURGE"

The idea that oestrogen may act to elicit LH release originated with the findings of Hohlweg (106) who observed formation of corpora lutea in the ovaries of immature rats

following a single injection of oestrogen. In the rat, under normal conditions, a small amount of LH is secreted continuously, although a much larger quantity of LH, in synergism with FSH, is required to bring about ovulation and formation of corpora lutea. Since moderate amounts of oestrogens were found to increase LH output from the pituitary, it was assumed that the high output of oestrogens during the preovulatory period might be responsible for the liberation of a preovulatory quota of LH (68). In addition, Everett (56) succeeded in hastening ovulation in normal cycling rats by a carefully timed injection of oestrogen. The site of positive feedback action of oestrogen on gonadotrophin release has not been definitely localized, though the findings that oestrogen implants into either the hypothalamus or POA induce ovulation or precocious puberty (199), suggest that these areas may be important sites with regard to this stimulation of gonadotrophin release by oestrogen.

Circulating oestrogen levels clearly and consistently increase several days before the LH surge in the Rhesus monkey (107) and in man (213). The preovulatory LH discharge in man (156, 213), Rhesus monkey (118, 146) and the rat (11) has not been found to be preceded by an increment in plasma progesterone concentration.

In the cycle of the rat it has been demonstrated that reduction of this rise in circulating oestrogen level, by administration of antisera to oestradiol but not to progesterone, effectively inhibits ovulation. The inference that this increase in circulating oestrogens may be the primary stimulus for the preovulatory LH discharge has been strengthened by the observation in sheep (81) and rats (32) that oestrogen administration leads to acute release of LH. Studies by Cross and Dyer (40) suggest

that the proestrous peak may be a response to declining oestrogen levels. In contrast to the striking inhibition of tonic LH secretion by the alpha-adrenergic blocking agents and by drugs with antidopaminergic activity, it has not been possible to block the preovulatory LH surge, whether spontaneous or induced, with these agents (122).

It is suggested that, in the Rhesus monkey, the negative and positive feedback actions of oestrogen on LH secretion appear to involve different neural pathways. The tonic secretion of LH and the cyclic secretion (surge) of LH seem to be controlled by different mechanisms, the one containing an adrenergic component which responds to low, relatively constant levels of oestrogen, the other responding to sustained increments in the plasma concentration of this hormone.

While the male pattern of LH secretion is believe to be of the tonic type and controlled in a similar manner to that of tonic LH secretion in the female, it has been found that oestrogen administration is unable to elicit LH secretion. Testosterone has been shown to block the LH release which occurs in response to oestrogen stimulation in the female rat (120).

Progesterone

There is considerable evidence in favour of the view that one of the main effects of progesterone is to suppress LH secretion (57). On the other hand, progesterone hastens ovulation in cyclic oestrous rats (60), constant oestrous rats (55), the cow (96) and the human female (180). The site of the positive feedback effect of progesterone on gonadotrophin release in rats has been localized in an area rostral to the MBH, and probably in the POA (207). More recent work by Mann and Barraclough (131) suggests that, not only do oestrogens and

progesterone act synergistically, but that adrenal progesterone might be of importance with regard to the positive feedback effect of this hormone on LH secretion.

Further evidence indicates that the positive feedback effects of both oestrogen (170, 187) and progesterone (60) on gonadotrophin secretion involve neural mechanisms which are subject to blockade by dibenamine (anti-adrenergic agent) and atropine (anti-cholinergic agent), thus implicating adrenergic and cholinergic pathways, respectively, in the feedback mechanism.

"Short Loop" Feedback

In addition to the feedback effects of the gonadal steroids on the higher centres of control, it is now generally accepted that "short" loop feedbacks exist whereby the pituitary hormones are able to control their own secretion by influencing hypothalamic function. Evidence in favour of such feedback loops stems from the observations that intravenous injection of LH is found to exert specific depressant effects on neuronal activity in the ventromedial hypothalamic nucleus of rats (173), as well as in the anterior hypothalamic and preoptic areas (211). Studies involving the implantation of FSH (mixed with cholesterol) near the median eminence in immature female rats resulted in decreases in both stalk-median eminence content of follicle stimulating hormone-releasing hormone (FRH) and in pituitary content of FSH (38), while prolactin implants in the same regions inhibited both the synthesis and release of endogenous hormone (34, 36).

SITE OF FEEDBACK ACTION OF STEROIDS IN THE BRAIN

In the rat, cyclic release of gonadotrophins does not occur in certain situations (225). These include prepuberal animals of both sexes, adult males, females with lesions of the medial preoptic area, animals with anterior deafferentation of the hypothalamus eliminating connections between the POA and MBH, females treated neonatally with androgens, pregnant animals and those exposed to constant light. However, ovulation can be made to occur in animals rendered acyclic by constant light exposure or early androgen treatment, by electrical stimulation of the POA, the median eminence or the arcuate nucleus (210). There is thus a close interrelationship between the neural and endocrine components in the control of gonadotrophin secretion. In order to define those areas within the brain concerned with the feedback effects of gonadal steroids, studies undertaken by various workers have included

a) implantation of gonadal steroids into the brain;
b) examination of different parts of the brain following administration of radioactively labelled gonadal steroids;
c) electrophysiological and ablative studies.

a) Steroid binding sites in the brain

Implants of oestradiol into the anterior hypothalamic or medial preoptic areas result in significant advancement of the time of vaginal opening, and of the initiation of normal ovarian cycles (200). While systemic effects of oestrogen may have some direct influence on the pars distalis, the onset of puberty due to oestrogen action at that site seems unlikely. Work by Dörner and Döcke (53) suggests that oestrogen, in promoting gonadotrophin secretion, increases hypophysial

sensitivity to the gonadotrophin releasing factors.

Kato and Villee (115) have supplied evidence for specific oestradiol-binding receptors in the pars distalis and anterior hypothalamus of the rat. Furthermore, the study demonstrated that the oestradiol was retained longer by these structures than by either the cerebral cortex or cerebellum, and that the oestradiol was retained as such and not metabolized during the 4-hour duration of the study. Work by McGuire and Lisk (137) suggests that the hypothalamus and pituitary gland are able to retain labelled oestradiol for at least 6 hours.

More recently, Orias and co-workers (162) have studied the effect of oestradiol, injected into the third ventricle of ovariectomized rats, on LH release. Following the injection of 5ng of oestradiol benzoate (Eb) into these animals, plasma LH levels decreased by 50% in 2 hours as compared with control animals. The same dose of Eb administered systemically had no effect upon LH release. In addition, pituitary sensitivity to intravenous injection of 100ng of LRH was not altered by the intraventricular injection of Eb. Since systemic administration of 100ng oestradiol, 3 hours prior to intravenous injection of 100ng LRH, decreased pituitary sensitivity to LRH (155), these findings suggest that the negative feedback action of oestradiol acts at both the pituitary and hypothalamic levels. (No alteration in FSH release was noted following the intraventricular injection of Eb.)

b) <u>Effects of steroids on brain activity</u>

In an investigation of the effects of oestrogen on unit firing rates in the septum, POA and hypothalamus of rats, Lincoln and Cross (127) noted inhibition of activity in the POA and anterior hypothalamus. Ramirez and co-workers (173) noted

that systemic administration of progesterone was followed by marked inhibition of the electroencephalogram, with moderate inhibition of unit activity in the hypothalamus. These findings were supported by the results of Arai and co-workers (4). Ovariectomy was found to result in a marked increase in sensitivity of individual neurones to progesterone after 2 to 3 weeks, an effect which could be prevented by 3-day oestrogen treatment prior to the progesterone injection (173). Furthermore, Cross and Kitay (41) reported increased neuronal activity in hypothalamic islands prepared with the Halasz knife as soon as 5 hours after ovariectomy.

All these findings suggest that gonadal steroids are able to exert a significant "damping down" effect on neural activity in the hypothalamus.

c) Ablative studies

Possibly one of the earliest reports of atrophy of the reproductive organs following operative damage to the hypothalamus in the region of the pituitary stalk was presented by Aschner (8), in studies with dogs. This initial finding has since been confirmed in the cat (186), sheep (35), rabbit (185), rat (200) and guinea-pig (46).

In addition, Dey (46) observed that, whenever the vaginal membrane remained closed and the ovaries were atrophic, following operation, the lesions had destroyed a large portion of the median eminence. Studies by Hillarp (104) on rats demonstrated that lesions placed anterior and ventral to the paraventricular nuclei resulted in constant oestrus, and it was suggested that this was due to the interruption of a fairly well demarcated fibre system running superficially on both sides of the median eminence towards the hypophysial stalk.

There has long been speculation about the ability of an island of hypothalamic tissue to maintain the various functions of the pars distalis. Szentagothai and co-workers (206), using stereotaxic surgical procedures, succeeded in producing this type of preparation. When the MBH was neurologically dissociated from the remainder of the brain, gonadotrophin secretion continued. The extent of the cut was such that most of the hypophysiotrophic area (i.e. the MBH adjacent to the infundibulum) was included within the island. Female rats with hypothalamic islands showed follicular development though ovulation was abolished. With incomplete rostral cuts, ovulation was not prevented, while bilateral cuts caudal to the optic chiasma blocked ovulation and led to persistent vaginal cornification. Cyclic ovulation, however, was not prevented if the rostral limit of the cut extended anteriorly to include the MPOA (93). The above information suggests that cyclical secretion of gonadotrophins (pre-ovulatory surge) is regulated by centres in the POA of the hypothalamus.

It has been proposed that the MPOA might be capable of autonomous activity in cyclically driving the hypophysiotrophic area, and might even be the seat of positive feedback of ovarian steroids essential for the "spontaneous" ovulatory discharge of gonadotrophin. Tejasen (209), studying the preopticotuberal pathway, obtained results suggesting a partial decussation of fibres in the retrochiasmatic region, such that a unilateral preoptic stimulus is distributed to the two sides of the pars distalis. The data support the hypothesis that an approximate point-to-point relationship exists between the MPOA and the hypophysiotrophic complex. Available evidence also suggests that the posterior hypothalamus might also play a role in the control of ovulation in the rat (59).

Neural substrate for the control of gonadotrophin secretion

Early evidence for the participation of catecholamines in the regulation of gonadotrophin secretion was presented by Sawyer and co-workers (188) who demonstrated that dibenamine, an adrenergic blocking agent, would inhibit ovulation in the rat. This initial finding was supported by the observed increase in monoamine oxidase activity in states of increased gonadotrophin secretion (227), and also by the demonstration of maximal hypothalamic content and turnover of catecholamines at, or just prior to, ovulation (50).

The anatomical substrate for adrenergic control of gonadotrophin secretion has been localized, by the use of fluorescent microscopy, to catecholaminergic and serotoninergic nerve endings in the hypothalamus (73). The findings of McCann and co-workers (135) suggest that dopamine is able to stimulate both FRH and LRH release in vivo and in vitro. A dopaminergic pathway, with cell bodies in the arcuate nucleus and axons projecting to the median eminence has been suggested as being responsible for the discharge of gonadotrophin-releasing factors (135). This arcuate nucleus-median eminence dopaminergic tract may be involved in the tonic secretion of gonadotrophins and in mediating the negative feedback action of the gonadal steroids. (Evidence suggests that oestrogen is able to block the response of the LRH-secreting neurones to dopamine). The authors believe that the positive feedback effects of the gonadal steroids are mediated more rostrally in the suprachiasmatic and preoptic regions - this view is based upon the observations that suprachiasmatic lesions block both ovulation and the progesterone-induced elevation of gonadotrophin secretion (134), while oestrogen implants into

this region advance puberty in the rat (199). Furthermore, lesions which separate the preoptic area from the remainder of the hypothalamus also block ovulation (94).

On the basis of these findings, supported by pharmacological studies involving progesterone-induced release of gonadotrophin (135), it appears that

a) noradrenergic pathways, extending from the anterior hypothalamic regions to the median eminence, are involved in mediating both the positive feedback effects of the gonadal steroids, and the cyclic pre-ovulatory release of gonadotrophin; while

b) the arcuate nucleus-median eminence dopaminergic pathways are involved in the negative feedback effects of the gonadal steroids and in the regulation of tonic release of gonadotrophin.

EFFECTS OF AUDIBLE SOUND ON REPRODUCTIVE FUNCTION

The effects of audible sound on the reproductive system have been investigated in common laboratory animals and, to a limited extent, in man. The physical characteristics of the sound stimuli used by the various workers (e.g. frequency, intensity and on-off sequence) differed widely and, in order to preserve some form of continuity in the following review, a brief summary of the sound parameters used by these workers is presented:

Zondek and Tamari (229)

Electric alarm bell
Stimulus applied 1 min in every 10 mins, day and night
Frequency of sound 3,000 - 12,000 Hz
Intensity of sound 70 - 110 db
Light intensity and light/dark ratio not specified

Singh, Cavanagh and Rao (197)

Audiogenerator-amplifier-speaker unit
Continuous sound stimulus, day and night
Frequency of sound 2,000 Hz
Intensity of sound ±100 db
Light/dark ratio kept constant by interval timers

Sackler and associates (183, 184)

Electric buzzer
Sound stimulus applied for either 1 min or 5 mins daily
Frequency of sound 375 - 500 Hz
Average intensity of sound 110 db

Arvay and associates (6, 7)

Electric alarm bell
Stimulus applied for 5 mins every hour
Frequency of sound 1,000 - 2,000 Hz
Intensity of sound 95 phone
Illumination 24 hours daily using reflector light of intensity 1,900 - 2,000 Lux
In addition, for 10 mins every day the animals were exposed to a combination of
a) intense light - 1,250 Lux
b) intense sound - 5,000 to 1,000 Hz; 105 - 115 phone
c) electrical stimuli - 70-80 V; 35-40 mA

This is described as the "program of intense cortical trauma".

Meyer (140)

Audiogenerator-amplifier-speaker unit
Sound applied for 1 min in every 10 mins
Frequency of sound 6,000 Hz
Intensity of sound 80 - 90 db

Henkin and Knigge (103)

Source of sound not specified
Sound applied continuously
Frequency of sound 200 - 220 Hz
Intensity of sound 130 - 135 db

STUDIES WITH SEXUALLY MATURE ANIMALS

a) Effects on Gonadal Function

Zondek and Tamari (229) exposed 30 adult female rats (180-200 grams) to 1 minute of audiostimulation in every 10 minutes, day and night. After 9 days of such treatment, histological examination of the ovaries revealed essentially normal follicles and corpora lutea. However, after 1 to 2 weeks, the rats displayed prolonged or persistent oestrus, the latter state being defined as at least 10 consecutive days of fully cornified vaginal smears. (In similar studies by Hagino (90) such changes first appeared only after 25 days of sound treatment.) After 60 days of audiostimulation there occurred an increase in uterine weight (33%), increased ovarian weight (60%), ovarian hyperaemia and a reduction in the number of mature follicles per ovary. Maturation of the follicles was not entirely arrested, as shown by the preservation of ova (228). Furthermore, the ovaries revealed a predominance of corpora lutea - up to 20 in some instances, as opposed to 6 or 8 in the ovaries of the control animals - while endometrial examination revealed an atypical proliferative pattern (228). These studies

were repeated using adult male rats in which similar exposure to the audiostimulus produced no detectable effects upon either testicular weight or spermatogenesis (230).

The apparently positive effect of audiostimulation on gonadal function was most convincingly demonstrated by Zondek and Tamari (229) using 48 adult female rabbits which had been kept isolated for at least one month prior to commencement of the experiment. Changes observed following 2 weeks of audiostimulation included a 2-fold increase in ovarian weight, follicle haematomas and increased formation of corpora lutea, the latter effect simulating the findings of a positive Friedman pregnancy test. In 6 of 16 such cases characterized by abundance of corpora lutea, enlargement of the mammae and galactorrhoea were induced by audiostimulation. Zondek and Tamari concluded that the morphological changes evoked by sound stimulation, which were similar to those induced in the rabbit by coitus, implantation of anterior pituitary tissue or intravenous administration of pregnancy urine gonadotrophin, represented manifestations of increased gonadotrophic activity of the anterior pituitaries of the animals studied (229, 231).

Singh, Cavanagh and Rao (197) subjected adult female rats to continuous audiostimulation throughout the day and night and noted that, after a variable period of irregular cycles, the treated rats displayed persistent oestrus, the highest incidence (66.6%) occurring after 150 days of such treatment. The early stages of audiostimulation were associated with irregular vaginal smears and ovaries containing follicles and/or corpora lutea (198), while prolonged exposure evoked the classical picture of persistent oestrus with polycystic ovaries containing no corpora lutea. The authors

interpreted these findings as being indicative of 2 phases of gonadotrophin secretion following commencement of the sound treatment: an initial phase of uninhibited release of LH, explaining the sustained corpora lutea, followed by decreased gonadotrophin secretion, responsible for the polycystic, atrophic state of the ovaries. Both uterine and ovarian weights increased significantly in response to the sound treatment, thus confirming the original findings of Zondek and Tamari (229).

Sackler and co-workers (183) studied the effects of both 1 minute and 5 minutes of daily audiostimulation, over a period of 11 consecutive days, on endocrine organs in adult female Wistar rats. A significantly greater response was noted in the group subjected to 5 minutes treatment per day, as compared with the 1 minute group. Other responses to audiostimulation included decreased rate of body weight gain (19%) and decreased liver weight (11.5%). In addition, appetite was severely affected and food consumption greatly reduced. While the gonadal effects reported by these workers are in direct opposition to those of Zondek and Tamari (229), it should be noted that these two, independent studies are in no way comparable due to differences in the source, physical characteristics and on-off sequence of the sound stimuli used. On the basis of their results, Sackler and co-workers concluded that sound stimulation exerted an inhibitory effect on the secretion of gonadotrophins and ovarian hormones.

Studies by Arvay (6), using his program of "intense cortical trauma", suggest the occurrence of two distinct phases in response to the treatment:

Early phase - Occurs within the first 10-12 days and is associated with more days of oestrus than in the corresponding time in the control rats. This phase is also characterized by accelerated maturation of the ovarian follicles, together with some well-defined degenerative changes associated with nerve plexuses.

Late phase - There now occurs an increasing time interval between the days of oestrus, with the cycle becoming irregular, until a constant diestrous pattern of vaginal smears is obtained after 42 days of audiostimulation.

In addition Arvay (6), using phosphorus-storing capacity of the ovary as an index of ovarian function, studied the distribution of P^{32} following audiostimulation. The specific activity of both pituitary and ovaries was found to increase by 100% only 30 minutes after a single neural trauma. With chronic exposure to sound this hyperactivity decreased somewhat, although still remaining elevated above normal levels even by the 42nd day of treatment. The conclusion was drawn that audiostimulation induces increased adenohypophysial secretion of gonadotrophins

Meyer (140), using a sound stimulus of frequency 6KHz and intensity 80db, noted depletion of neurosecretory material from the anterior hypothalami of sound-exposed rats. Similar depletion of neurosecretory material from the supraoptic and paraventricular nuclei has been reported by Arvay (5, 6), following intense cortical trauma.

b) <u>Posterior Pituitary Effects</u>

Pure sound, in the form of recorded thunderclaps of

frequency 150 Hz and intensity of 98-100 db, has been found to induce diuresis, kaliuresis and natriuresis in rats, and is associated with an increase in plasma oxytocin level (159, 160). These urinary changes, similar to those induced by subcutaneous injection of 4mU of oxytocin could not be demonstrated in neurohypophysectomized rats, although the kaliuretic effect in these rats could be induced by administration of oxytocin. The authors believe the effects to be due to secretion of oxytocin and, to a lesser extent, of antidiuretic hormone.

Deis (45) reported increased milk ejection in lactating rats when under the influence of auditory stimuli produced by a mother and young while nursing, the magnitude of this effect being equivalent to the administration of 20mU of oxytocin/100g body weight. Milk ejection in deaf lactating mothers was uninfluenced by the auditory stimuli, suggesting that the stimuli evoke release of oxytocin.

c) Thyroid Effects

Arvay and co-workers (7) suggest that intense nervous stimuli induce a state of thyroid hyperfunction after about 10 days, followed by hypofunction. The effects are reversible and the response may be inhibited by chlorpromazine. Studies by Sackler and co-workers (183) have shown a 2.5% increase in thyroid weight in rats subjected to 5 minutes of audiostimulation per day, over a period of 11 consecutive days. The effect was similar in the 1 minute group (4.8% increase), although the number of rats displaying this effect was greatly reduced.

d) Adrenal Effects

Although Zondek and Tamari (229) observed the occurrence of adrenal hyperplasia in their sound-exposed rats, they disregarded this as being indicative of a systemic stress reaction, the latter being expected to induce gonadal atrophy. Consistent adrenal hyperplasia is reported by Sackler and co-workers (183), who noted a 13.9% increase in adrenal weight following sound stimulation. Elevated adrenocorticoid secretion is also suggested by a decrease in circulating eosinophils, increased glutathione levels, increased serum and adrenal cholesterol and increased adrenal ascorbic acid in sound exposed rats (7, 51, 103).
Henkin and Knigge (103) found that the stress of continuous high intensity sound (frequency 220Hz and intensity 130 db) evoked an adrenocortical response in rats, characterized by an initially high rate of corticoid secretion, followed by depression of secretion and then elevation again to high levels. The period of suppressed corticoid output appeared to depend upon 12 hours of continuous sound stimulation, the general response pattern being abolished following bilateral surgical destruction of the middle ear. The authors suggest that the suppression of corticoid output occurs secondary to inhibition of adrenocorticotrophic hormone (ACTH) secretion.

e) Effects on Fertility

In apparently marked contrast to the stimulating effect of audiostimulation on female genital function is the inhibitory action on fertility. Studies by Zondek and Tamari (230) reveal a fertility rate in control rats, over a period of 14 months, of average 78%. Audiostimulation prior to copulation

was found to reduce this figure dramatically in both male and female rats:

Male fertility: Forty adult male rats were exposed to sound stimulation for a period of 2-8 weeks and allowed to copulate in the subsequent 2-3 weeks with unexposed females. Of 40 females, only 3 became pregnant - i.e. fertility rate of 7.5%. In a further study the rats were exposed to sound for 9 days and allowed to copulate during the following 4 days. Of 80 males so treated, only 9 were able to fertilize females - i.e. fertility rate of 11.2%. No changes were detected in either the weights of the testes and seminal vesicles, or in histological studies of the spermatogenic process. In addition, the finding of sperm ejaculate in the vagina of the female partner (usually within the first 24 hours of the copulation period) suggested that the males had neither lost their sexual potency nor been refused by the females. Similar findings were reported by Sackler and associates (184) who exposed adult male rats to a single 5 minute period of audiostimulation per day for 3 weeks. Histological examination revealed no change in the germ cells over a wide range of sound frequencies employed.

Female fertility: Zondek and Tamari (230) repeated the study, this time exposing 50 females to 9 days of audiostimulation, and allowed them to copulate with unexposed males over the subsequent 4 day period. The females were sacrificed 20 days after the first day of copulation. Of the 50 females studied, only 9 proved to be fertile - fertility rate reduced from 70-80% to 18%. As in the case of the males studied, the presence of sperm ejaculate in the vagina of the female confirmed that copulation had indeed occurred, and no anatomical changes in the ovaries were detected which could explain the

infertility. The infertility induced by auditory stimuli was not improved by a rest period of 2-4 days following the 9 days of treatment. With an 8 day rest period, fertility improved to 50% and only after 12 days did the rats regain their normal fertility.

In order to test the hypothesis of Sackler and associates (183) that audiostimulation inhibits gonadotrophic function of the adenohypophysis, Zondek and Tamari (230) administered pregnant mare serum gonadotrophin (PMSG), with mostly FSH activity, and human chorionic gonadotrophin (HCG), with mostly LH activity, during the 9 days stimulation period. Ten female rats, subjected to 9 days of audiostimulation, were injected subcutaneously with 5IU of PMSG on day 8, given 2 days of rest and allowed to copulate with unexposed males for 4 days. The females were sacrificed 20 days after the first day of copulation. Fertility rate was reduced from 70-80% in control rats to 10% in the treated animals. Administration of 5IU of HCG produced similar results. Separate studies with males and females, involving administration of 5IU PMSG and 5IU HCG on days 8 and 10 of the stimulation period respectively, showed a decline in fertility rate to 20%. Thus the deleterious effect of audiostimulation preceding copulation on fertility could not be altered by treatment with PMSG or HCG during the stimulation period, thus adding further weight to the hypothesis of sound-induced stimulation of gonadotrophin secretion (229).

Despite reports that auditory stimuli evoke rapid discharge of ACTH with adrenal ascorbic acid depletion, hypertrophy of the adrenal cortex and stimulation of

corticosteroid genesis (7, 51, 103, 183, 229), Zondek and Tamari (230) investigated the effects of corticosteroid administration on the reduction in fertility consequent to audiostimulation. In studies with both males and females, injection of 0.05mg hydrocortisone acetate on days 7 and 8 of the 9 days audiostimulation period, could not prevent a reduction in fertility rate to 20%. The fertility reduction was also unaffected by replacement of the drinking water by saline, and by daily injection of 2 or 4mg alpha tocopherol acetate throughout the stimulation and copulation periods. Seasonal variation also had no effect upon the response to audiostimulation.

STUDIES WITH HUMAN SUBJECTS

In addition to the reported effects of audiostimulation on reproductive function in animals, a modest volume of literature is available which suggests that audiostimulation is capable of enhancing circulating gonadotrophin levels in man.

Meyer (140) has presented evidence suggesting that pure sound stimuli are effective in inducing ovulation in women with certain anovulatory conditions associated with hyposecretion of gonadotrophins. Beardwood (14) studied urine total gonadotrophin activity (TGA) in 5 normal male subjects before, during and after exposure, for 1 minute in every 10, to an 85 db /6KHz audiostimulus. This treatment lasted for 2 hours on each of 3 consecutive days. In 3 subjects a significant increase in TGA output occurred after 2 days of such treatment, while 3 days of auditory stimulation evoked mean TGA responses in all subjects, which were greater than those following 2 days of

treatment and markedly differed fro m control values. Furthermore, Beardwood et al (15) have provided information suggesting that audiostimulation influences, in a characteristic manner, the pulsatile pattern of LH secretion in normal men. Recent studies by Beardwood et al (16) have revealed maximum urinary LH concentrations in normally menstruating subjects after 3 daily sessions of audiostimulation. Results suggest that the peak urinary LH concentrations induced by audiostimulation are distinguishable from the normal ovulatory peak.

C H A P T E R 2

THE MEASUREMENT OF RAT SERUM LH CONCENTRATION BY DOUBLE ANTIBODY RADIOIMMUNOASSAY

Chapter 2

THE MEASUREMENT OF RAT SERUM LH CONCENTRATION BY DOUBLE ANTIBODY RADIOIMMUNOASSAY

Prior to the development of radioimmunoassays for protein and polypeptide hormones, the biological methods employed were not only expensive, laborious and imprecise, but also lacked sensitivity, see (20). Originally employed for the immunoassay of insulin (224), radioimmunoassays have since been adapted for the measurement of nanogram and picogram quantities of many substances present in biological fluids to which specific antibodies can be raised. More recently it has become possible to extend the immunoassay method to compounds such as the steroid hormones (143) which are intrinsically non-antigenic. "The concentrations of protein hormones in plasma do not generally exceed 10^{-10}M when the secreting gland is at rest, but may increase 10 to 50 fold following appropriate physiologic stimulation or in the presence of a hormone-secreting tumour. Since the concentration of serum proteins is of the order of 10^{-3}M, it is evident that both a high degree of sensitivity and a high degree of specificity are required for detection and measurement of protein hormones in plasma." (20) The development of the modern radioimmunoassay technique has enabled such levels of sensitivity and specificity to be reached with a high degree of accuracy, and has made possible the measurement of low levels of gonadotrophin to be carried out in physiological fluids (158, 172).

The production of purified rat pituitary hormones and specific antibodies to these hormones by the National

Institutes of Health (NIH) in America, has prompted considerable research activity into the physiology of pituitary hormones.

Specific radioimmunoassays for rat pituitary LH have been developed by Niswender et al (158), while Naftolin and Corker (152) recently published a microassay technique for rat LH. The latter has been modified in this study for use with the NIH reagents.

MATERIALS

(A) BUFFERS

1) 0.5M Phosphosaline Buffer (0.5M PSB)
0.15M NaCl; 0.5M Na_2HPO_4 (pH 7.5)
(pH adjusted by addition of 0.5M KH_2PO_4 in 0.15M NaCl)

2) 0.01M Phosphosaline Buffer (0.01M PSB) - for elution of Sephadex and Cellulose columns and as sample diluent.
(1/50 dilution of above 0.5M PSB with 0.15M NaCl) pH 7.4

3) 0.01M Phosphosaline Buffer with ethylenediamine tetraacetic acid (0.01M PSB EDTA) - diluent for antibodies and labelled LH.
(0.15M NaCl, 0.01M Na_2HPO_4 buffer containing 0.002M EDTA) pH 7.6

4) 0.01M PSB EDTA containing 1% normal rabbit serum (NRS) for dilution of precipitating antiserum (second antibody).

5) 0.01M PSB EDTA containing 4% human serum albumin (HSA) for dilution of LH antiserum and iodinated (labelled) LH.

6) 0.01M PSB containing 4% HSA - for dilution of serum or plasma.

7) 0.01M PSB containing 5% HSA - for use with gel filtration columns.

(B) HORMONES and ANTISERA

1) LH Antiserum (Anti-LH or "first antibody")

NIAMD-Anti-Rat LH Serum-1

Dispensed in 0.1ml aliquots, as a 1/100 dilution with 0.01M PSB containing 1% NRS, into capped tablet vials (Apex Tablet Vials, SA Druggists Ltd), snap frozen in dry ice and acetone and stored at -18°C.

2) Precipitating Antiserum)Anti-rabbit gamma globulin - ARGG or "second antibody")

Anti-rabbit Precipitating Serum (donkey), Wellcome Reagents Ltd.

3) Tracer LH NIAMD-Rat LH-1-1

Diluted with 0.01M PSB such that 10ul contains 2.5µg of hormone. Dispensed in 15µl aliquots into ACTH assay tubes (ACTH Immunoassay Kit, Radiochemical Centre, Amersham,) snap frozen in dry ice and acetone and stored at -18°C.

4) LH Standard NIAMD-Rat LH-RP-1

1mg was diluted in 0.01M PSB containing 0.5% HSA such that 1ml contained 1µg of hormone. Dispensed in 1ml aliquots into tablet vials, snap frozen in dry ice and acetone and stored at -18°C.

5) Internal Serum Pools consisted of sera derived from

a) intact female rats

b) bilaterally ovariectomized rats

The sera were dispensed into assay tubes (Thomas Micro Centrifuge Tubes, Thomas Scientific Apparatus, in 50µl aliquots and stored at -18°C. The total number of stored

aliquots was sufficiently large to last throughout the course of this study and were included in each assay to provide an index of interassay variation.

6) Specimens consisted of serum (or plasma in the case of heparinized blood) derived from freshly centrifuged whole blood which had been allowed to clot at room temperature for at least 1 hour following collection. The blood samples were centrifuged at 3,000rpm for 10 minutes and the serum or plasma removed with disposable glass pipettes (Medglass Pasteur Capillary Pipettes). All samples were stored at -18°C while awaiting LH radioimmunoassay.

(C) REAGENTS for IODINATION PROCEDURE

1) 0.5M PSB (20µl)

2) NIAMD-Rat LH-1-1 2.5µg (10µl)

3) Chloramine-T (E. Merck AG Darmstadt)
12.5mg in 5ml 0.01M PSB
(Prepared immediately prior to use)

4) Sodium Metabisulphite (May and Baker Ltd)
10mg in 5 ml 0.01M PSB
(Prepared immediately prior to use)

5) Sodium ^{125}Iodide (Radiochemical Centre)
500µCi (5µl)

6) 0.01M PSB containing 5% HSA

7) Sephadex G-50 fine (Pharmacia Fine Chemicals)
in 0.01M PSB pH 7.4

8) CF 11 Fibrous Cellulose Powder (Whatman Column Chromedia)
in 0.01M PSB.

M E T H O D S

IODINATION of RAT LH

The iodination procedure was carried out in 4 stages, the actual reaction and elution of reaction components being performed in a fume cupboard behind an 18cm high wall of lead bricks. The stages involved were as follows:

1) Preparation of chromatography columns;
2) Iodination reaction;
3) Separation of labelled LH from free iodine^{125} on Sephadex G-50 column;
4) Further purification of labelled LH on a cellulose (CF 11) column.

1) Preparation of Columns

Sephadex G-50: A gel filtration column of internal diameter 9 mm was filled to a height of ±10cms with Sephadex G-50 (fine) in 0.01M PSB and eluted with the same buffer. Once the Sephadex had packed firmly, the "active sites" on the column were saturated by the addition of 2 to 3 ml of 0.01M PSB containing 5% HSA. The column was then washed with 0.01M PSB until a negative Albustix test was obtained from the eluate. With the buffer meniscus about 0.5cm above the Sephadex face, the outflow of the column was stopped.

Cellulose (CF 11): A column, similar to that used in the preparation of the Sephadex column, was filled to a height of ±7cms with CF 11 suspended in 0.01M PSB. The column was washed with 0.01M PSB and the outflow of the column

occluded when the buffer level was 0.5cms above the face of the cellulose.

2) Iodination Reaction

The Chloramine-T method of Hunter and Greenwood (110), Greenwood et al (86), was used in the preparation of iodinated rat LH. The reaction was performed in an Auto Analyzer Vial (Sterilin Ltd) into which the components of the reaction mixture were added in the following sequence:

a)	$Na^{125}I$	500µCi contained in	5µl
b)	0.5M PSB		20µl
c)	NIAMD-Rat LH-1-1	2.5µg	10µl
d)	Chloramine-T	25.0µg	10µl
.....	reaction time 25 seconds		
e)	Sodium Metabisulphite	200.0µg	100µl

3) Separation of Labelled LH from Free $Iodine^{125}$

The entire contents of the reaction vial was applied to the Sephadex G-50 column and 0.5ml aliquots of eluate collected into each of 24 disposable tubes (Luckham LP3). The column was eluted with 0.01M PSB. Radioactivity in each tube was estimated in a well counter and expressed as counts/second. A typical elution pattern is shown in FIG ..1.. Since the components of high molecular weight are eluted first from the Sephadex column, Peak 1 represents iodine which is bound to LH, thus providing an index of specific radioactivity of the LH. Peak 2 represents free $iodine^{125}$.

4) Adsorption Chromatography on Cellulose

The labelled LH was further purified using the technique

of adsorption chromatography, first used for purification of ^{125}I-LH after iodination (109,204). The contents of those tubes represented by the apex and trailing shoulder of Peak 1 in FIG 1 were pooled and applied to the CF 11 column. The column was first eluted with 18ml 0.01M PSB, during which stage the intact, labelled LH was bound to the cellulose. 1.5ml aliquots of eluate, containing free iodine and peptide breakdown products, with traces of reaction mixture components, were collected into each of 12 tubes. Thereafter, the column was eluted with 5% HSA in 0.01M PSB, to displace the labelled hormone from the column. 0.7ml aliquots of eluate were collected into each of 12 tubes and the radioactivity of each measured as above. A typical elution pattern is shown in FIG 1 (Appendix TABLE 1)

The contents of those tubes represented by the apex and trailing shoulder of peak 4 were pooled, suitably diluted in 0.01M PSB EDTA containing 4% HSA to provide 10,000cpm of activity in 0.05ml, and added to the assay tubes. The specific activity of the labelled LH was always between 35 and 70μCi/μg.

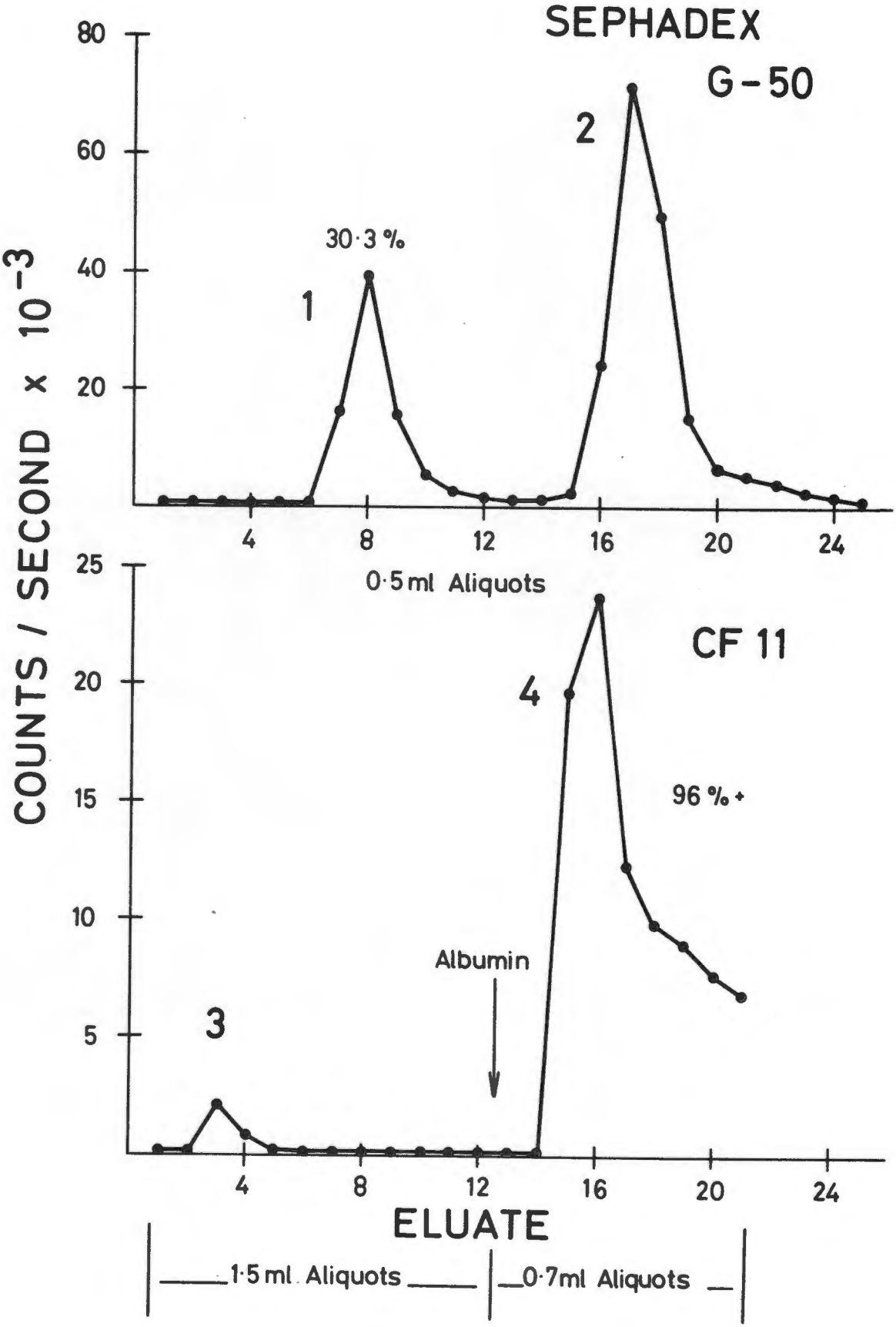


FIG 1 Iodination of Rat LH: Sephadex G-50 and Cellulose CF 11 elution patterns

THE ASSAY PROCEDURE

The assay, which extends over a period of 11 days, was performed in duplicate in capped microtubes (Thomas Micro Centrifuge Tubes - Thomas Scientific Apparatus), and required 50µl, per assay tube, of serum or plasma. With the exception of second antibody, which was added together with the normal rabbit serum in a single 100µl volume, and the unknown serum or plasma (50µl added with a Finnpipette - Jencons Scientific Apparatus), additions to the assay tubes were made in 50µl quantities using a 2ml Repette (Jencons Scientific Apparatus). The assay tubes, each containing a final volume of 250µl, were arranged in duplicate as depicted in TABLE ..1.. The day-to-day assay procedure was as follows:

DAY 1 (am)

The addition of 50µl of each of the following to the assay tubes:

a) 1/64,000 dilution of rabbit anti-rat LH serum (first antibody) in 0.01M PSB EDTA containing 4% HSA, to all tubes other than total count (TC) and non-specific binding (NSB) tubes.

b) Doubling dilutions of rat LH standard varying between 4 and 500 ng/ml to tubes 1 to 16 respectively, for future plotting of the standard curve. Dilutions were made using the 1,000 ngLH/ml stock standard solution and 0.01M PSB. (In order to facilitate plotting of the standard curve, tubes 7 and 8 in TABLE ..I.. , which would actually contain 31.25 ngLH/ml, were represented by the point on the curve corresponding to 32 ngLH/ml. Dilutions

TABLE ....1 Volumes of the respective solutions contained within the rat LH assay tubes.

	Tube	Buffer	Ab in (A)*	LH STD in (B)*	Label in (A)	NRS & ARGG
	TC	-	-	-	50µl	-
	NSB	50µl (A) 50µl (B)	-	-	50µl	100µl
	0%B	50µl (B)	50µl	-	50µl	100µl
4ng LH/ml	1-2	-	50µl	50µl	50µl	100µl
8ng LH/ml	3-4	-	50µl	50µl	50µl	100µl
16ng LH/ml	5-6	-	50µl	50µl	50µl	100µl
32ng LH/ml	7-8	-	50µl	50µl	50µl	100µl
62.5ng LH/ml	9-10	-	50µl	50µl	50µl	100µl
125ng LH/ml	11-12	-	50µl	50µl	50µl	100µl
250ng LH/ml	13-14	-	50µl	50µl	50µl	100µl
500ng LH/ml	15-16	-	50µl	50µl	50µl	100µl
NSB Unknown		50µl (A)	-	50µl serum pool	50µl	100µl
Unknown		-	50µl	50µl serum	50µl	100µl

*(A) = 0.01M PSB EDTA containing 4% HSA

*(B) = 0.01M PSB

were made using the 1,000 ngLH/ml stock standard solution and 0.01M PSB.

c) 50µl of serum or plasma to be assayed to tubes labelled "unknown". Where necessary, dilution of unknown serum or plasma samples was performed using 0.01M PSB containing 4% HSA.

d) 50µl of pooled unknowns (consisting of equal volumes of each unknown which are mixed) added to tubes labelled "NSB unknown" - these were included as an index of non-specific binding of label to the unknown serum or plasma.

e) Suitable buffer to bring the volume contained in each tube to 100µl, while maintaining constant the quantity of human serum albumin in each tube.

f) Pooled rat serum - tubes containing 50µl serum were included in each assay to provide an index of interassay variation.

The contents of each tube was thoroughly mixed using a Whirlimixer (Fisons Scientific Apparatus Ltd), and centrifuged at 3,000rpm for 3 minutes to remove the air bubbles. The tubes were then incubated at 4°C for 3 days.

DAY 4 (am)

Iodination of rat LH, in the manner described previously, was carried out on day 4.

50µl of labelled LH was added to each tube. Once again, the contents of the tubes were mixed, centrifuged and incubated at 4°C for 6 days.

DAY 10 (am)

Second antibody (ARGG) was added to the tubes in order to

precipitate the antigen-antibody complexes.

The solution was prepared as follows:

1 volume 1/8 dilution of ARGG in 0.01M PSB EDTA containing 1% NRS;

1 volume 0.01M PSB EDTA containing 1% NRS;

2 volumes 0.01M PSB EDTA.

100 µl of this solution was added to each tube, with the exception of the total counts tubes. The contents of the tubes were mixed, centrifuged and incubated at 4°C for 24 hours.

DAY 11 (am)

All the tubes other than the total counts tubes were centrifuged at 3,000rpm for 30 minutes (MSE Multex Centrifuge), after which the tip of each tube was held firmly and the supernatant fluid flicked towards the opposite end, away from the precipitate. The tubes remained inverted in a refrigerated tray while radioactivity of each tip was measured.

The tip of each tube,containing the precipitate, was cut off, inserted into a polythene counting tube (Luckham LP3, Luckham Ltd) and activity counted for 60 seconds using a well counter. Typical results and calculations are shown in TABLE ....2.

CALCULATIONS

The zero binding (0%B) tubes, which contain no unlabelled LH, should contain the highest number of counts and, after subtraction of the counts contained in the non-specific binding tubes, are equated to 100%. The counts in the remainder of the assay

tubes are then calculated as percentages of the counts in the zero binding tubes.

The standard curve is plotted on semilogarithmic graph paper and the LH values for the unknown samples obtained by interpolation.

The standard curve derived from the results in TABLE .2.. is shown in FIG .2..

TABLE ..2.. Results of a typical rat LH assay.

Tube	Counts/min		$\bar{x}$	$\bar{x}$ - NSB	(%TC) $\bar{x}$ - NSB	B/B_o	LH (ng/ml)
TC	9528	9416	9421				
	9154	9386					
	9616	9045					
	9912	9307					
NSB*	2009	2030	2020		21.4		
0%B	7769	7701	7735	5715	60.7		Standards
1-2	7663	7659	7661	5641	59.9	98.7	4
3-4	7483	7178	7331	5311	56.4	92.9	8
5-6	7046	7204	7125	5105	54.2	89.3	16
7-8	6617	6422	6520	4500	47.8	78.4	32
9-10	5760	5791	5776	3756	39.9	65.7	62.5
11-12	4554	4610	4582	2562	27.2	44.8	125
13-14	3511	3551	3531	1511	16.0	26.4	250
15-16	3227	3186	3207	1187	12.6	20.8	500
							Unknowns
NSB*	1752	1693	1723		18.3		
A	5146	5043	5095	3372	35.8	59.0	78
B	5466	5404	5435	3712	39.4	64.9	64
C	5691	5314	5503	3780	40.1	66.1	61

* The radioactivity of the NSB tubes consists of the true non-specific binding plus the background count. The latter was about 1600 counts/min. The true non-specific binding was of the order of 1% of total counts.

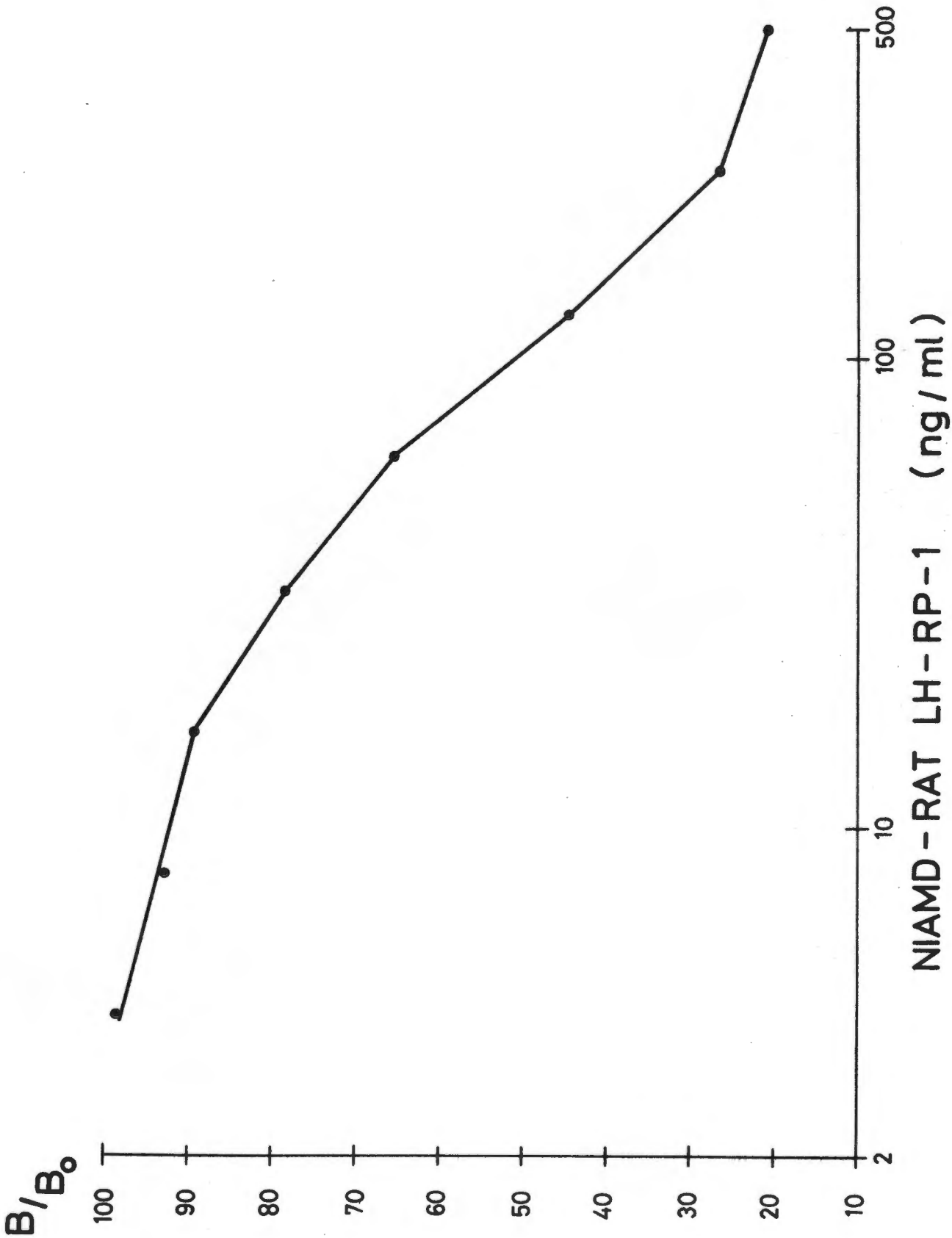


FIG 2 Rat LH Radioimmunoassay: Standard curve derived from the results shown in TABLE 2.

The modern radioimmunoassay technique, which is based upon the principle of competition between labelled and unlabelled hormone for binding sites on an antibody specific to that hormone, is both highly sensitive and subject to numerous physicochemical influences. It is therefore essential, when establishing a new radioimmunoassay technique or extending an existing technique to other hormones, for certain aspects of the assay procedure to be clearly established. These include

a) antibody dilutions
b) incubation times (durations of incubation)
c) reliability criteria

A N T I B O D Y D I L U T I O N S

First Antibody Titration

In order to determine the dilution of rabbit anti-rat LH serum required for optimal binding of labelled LH, a constant amount of the latter was added to each of a series of duplicate tubes containing doubling dilutions of the first antibody between 1/4,000 and 1/256,000.

The form of the antibody titration, which was performed in two separate assays, together with the contents of the tubes is shown in TABLE .3..

0.01M PSB EDTA containing 4% HSA was added to the appropriate tubes to maintain constant the colume of reaction mixture contained in each tube. Following addition of first antibody and the required volume of buffer, the tubes were incubated overnight at $4^{o}C$ before addition of 50µl of labelled LH to each. The labelled LH was diluted in the above buffer to

TABLE 3....

FIRST ANTIBODY TITRATION

A S S A Y 1

Tube	Buffer (µl)	Anti-LH Vol.	Anti-LH Dil.	Label (µl)	NRS & ARGG (µl)
TC	-	-	-	50	-
NSB	100	-	-	50	100
1-2	50	50	$1/4 \times 10^{-3}$	50	100
3-4	50	50	1/8	50	100
5-6	50	50	1/16	50	100
7-8	50	50	1/32	50	100
9-10	50	50	1/64	50	100

A S S A Y 2

TC	-	-	-	50	-
NSB	100	-	-	50	100
1-2	50	50	$1/32 \times 10^{-3}$	50	100
3-4	50	50	1/64	50	100
5-6	50	50	1/128	50	100
7-8	50	50	1/256	50	100

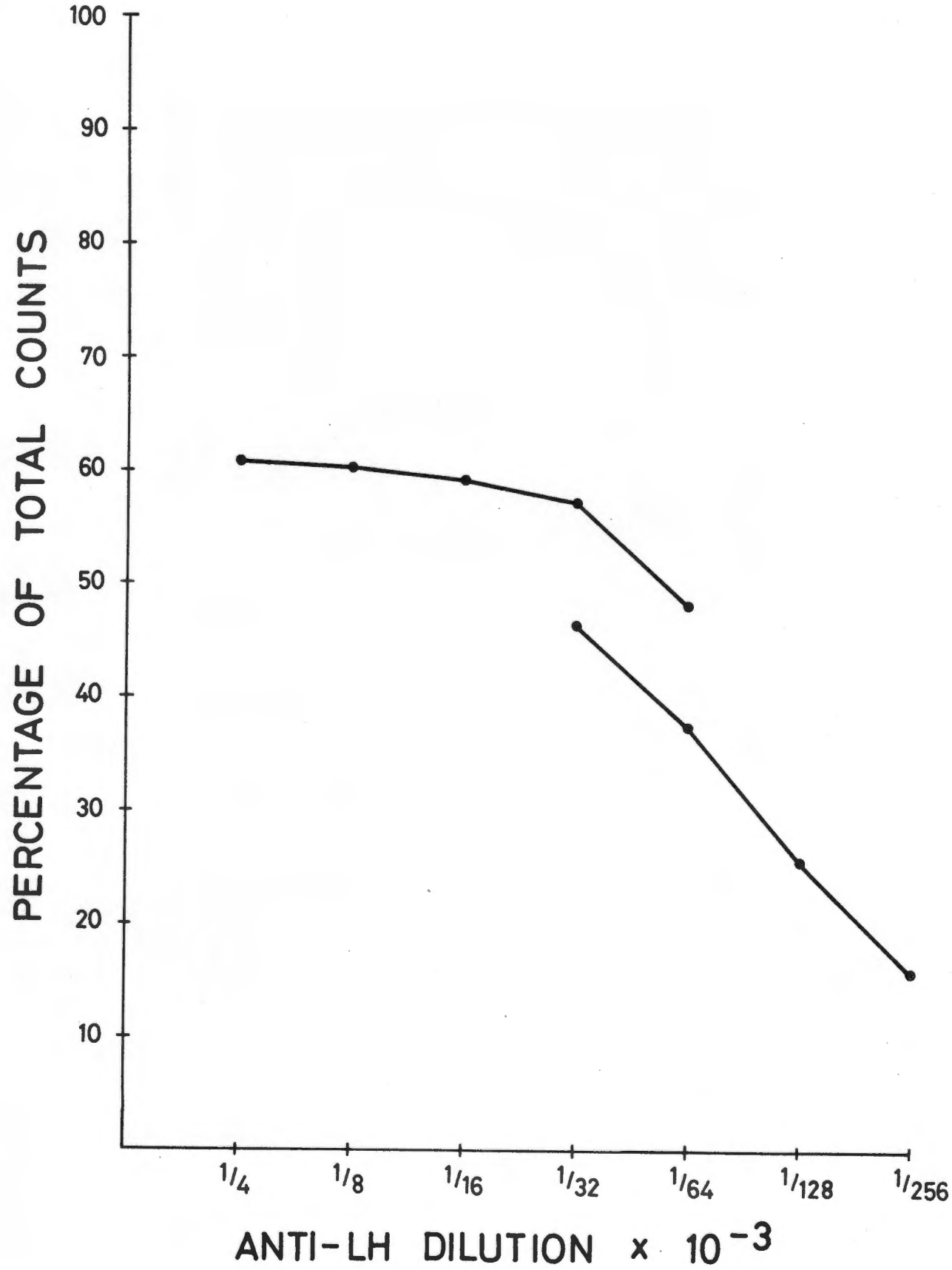


FIG 3 Rat LH Radioimmunoassay:
First antibody titration curves

provide 10,000cpm of activity in 50µl. Once label had been added, a second incubation period of 6 days at 4°C elapsed before addition of the second antibody as described above. A final incubation of 24 hours at 4°C supervened before centrifugation and counting of the precipitates for radioactivity (see page ..56)

Results are depicted graphically in FIG ..3.. (see Appendix TABLE ..2..) Maximal binding of labelled LH occurred up to a dilution of first antibody of 1/32,000, thereafter the curve dropped off steeply as progressively less of the labelled LH was bound.

At a first antibody dilution of 1/64,000 slightly less than 50% binding was obtained. On the basis of this finding a first antibody dilution of 1/64,000 was selected as being most appropriate for the assay system.

Second Antibody Titration

The procedure followed was essentially similar to that described for the first antibody titration, involving first, second and third incubation periods of 1, 6 and 1 days respectively (TABLE ..4..)

Since maximal binding of labelled LH to the first antibody was desired, the latter was added to the tubes as a 1/2,000 dilution with 0.01M PSB containing 1% NRS. Once again, label was suitably diluted with buffer to provide 10,000cpm of activity and added as a 50µl aliquot. After the second incubation period, doubling dilutions of NRS and ARGG (second antibody) were added as described in the first antibody titration method above. These dilutions varied between 1/4 and 1/64.

Results are shown in FIG ..4.. (see Appendix TABLE .3...)

More than 70% of the total counts added were precipitated by second antibody at a dilution of 1/8. Thereafter, radioactive precipitation declined rapidly at dilutions of antibody greater than 1/16. A 1/8 dilution of second antibody was selected for the assay system.

TABLE 4....

SECOND ANTIBODY TITRATION

Tube	Buffer (µl)	Anti-LH (µl) (1/2,000)	Label (µl)	Dilution of ARGG	NRS & ARGG (µl)
TC	-	-	50		-
NSB	100	-	50	1/4	100
1	50	50	50		100
NSB	100	-	50		100
2	50	50	50	1/8	100
NSB	100	-	50	1/16	100
3	50	50	50		100
NSB	100	-	50	1/32	100
4	50	50	50		100
NSB	100	-	50		100
5	50	50	50	1/64	100

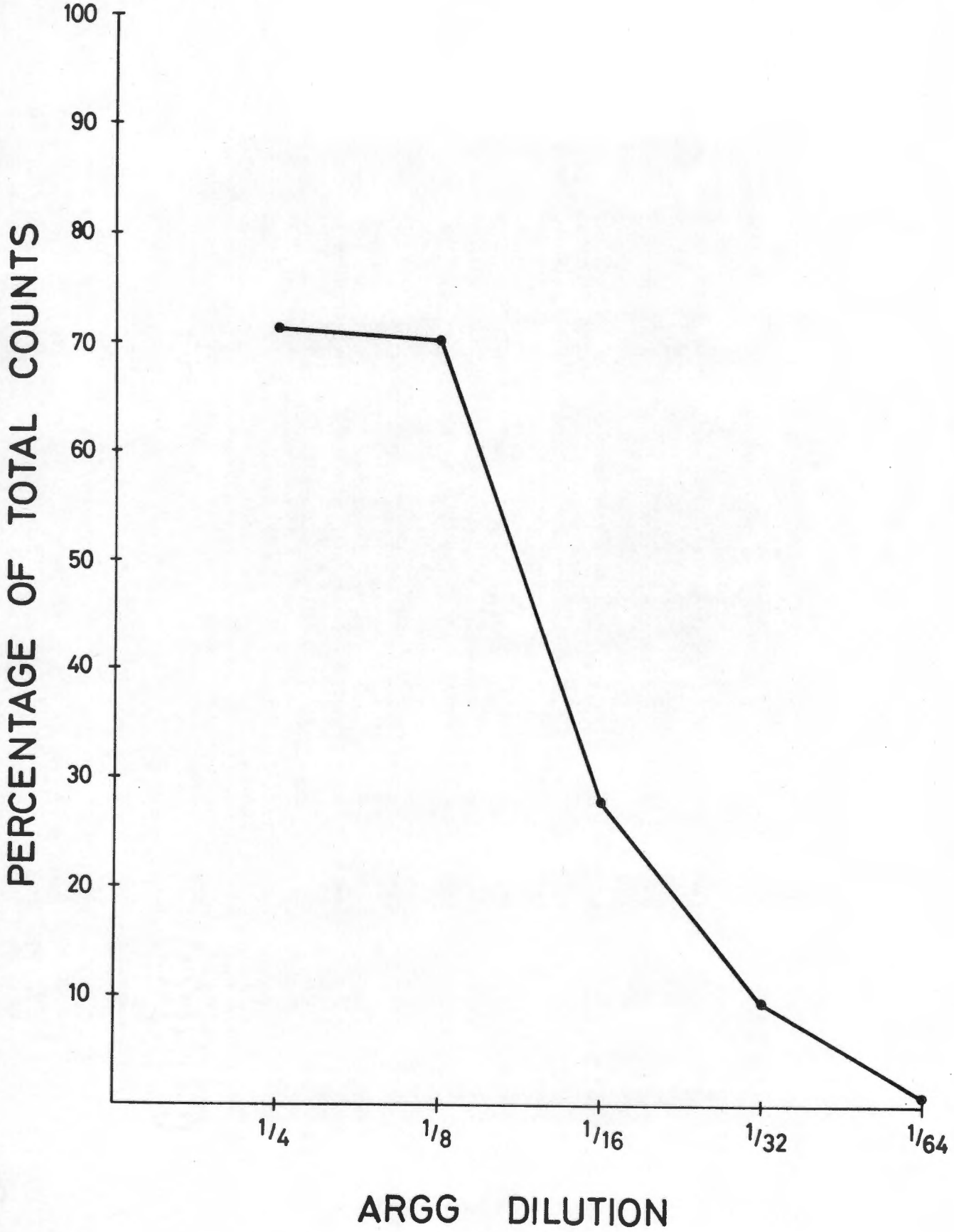


FIG 4 Rat LH Radioimmunoassay:
Second antibody titration curve

DURATION OF INCUBATION

First Incubation Time

In an attempt to determine the optimum duration of the incubation period necessary for maximal binding of LH to the first antibody, standard curves were set up as shown in TABLE .5.. The day-to-day procedure was as follows: Two standard curves, A and B, were prepared in series on each of 3 consecutive days. Curves A and B contained anti-rat LH (first antibody) dilutions of 1/32,000 and 1/64,000 respectively. On day 4, 50µl of labelled LH (containing approximately 10,000cpm of radioactivity) was added to each of the tubes before incubation at 4°C for 144 hours. An optimal second incubation period of 144 hours had been established.

The subsequent addition of second antibody, 24 hour incubation and centrifugation before counting was performed as described in the assay method (see page .56.) In this manner the standard curve tubes filled on days 1, 2 and 3 had undergone first incubation periods of 72, 48 and 24 hours respectively by the time labelled LH was added. The standard curves are shown in FIGS .5.,.6.,.7. (Appendix TABLE .....4.....)

In addition to demonstrating the form of the standard curve under conditions of varying first incubation time, the curves clearly display a shift to the left with increasing anti-LH dilution.

A first incubation period of 72 hours (with anti-LH dilution of 1/64,000) was selected as being most satisfactory.

TABLE ..5.

FIRST INCUBATION TIME

Tube	Buffer	Anti-LH in Buffer (A)	LH STD in Buffer (B)	Label in Buffer(A)	NRS & ARGG
TC	-	-	-	50µl	-
NSB	50µl of Buffer(A) +50µl of Buffer(B)	-	-	50µl	100µl
0%B	50µl of Buffer(B)	50µl	-	50µl	100µl
4 ngLH/ml	-	50µl	50µl	50µl	100µl
8	-	50µl	50µl	50µl	100µl
16	-	50µl	50µl	50µl	100µl
32	-	50µl	50µl	50µl	100µl
62.5	-	50µl	50µl	50µl	100µl
125	-	50µl	50µl	50µl	100µl
250	-	50µl	50µl	50µl	100µl
500	-	50µl	50µl	50µl	100µl

(A) = 0.01M PSB EDTA containing 4% HSA

(B) = 0.01M PSB

Anti-LH: 1/32,000 and 1/64,000 dilutions.

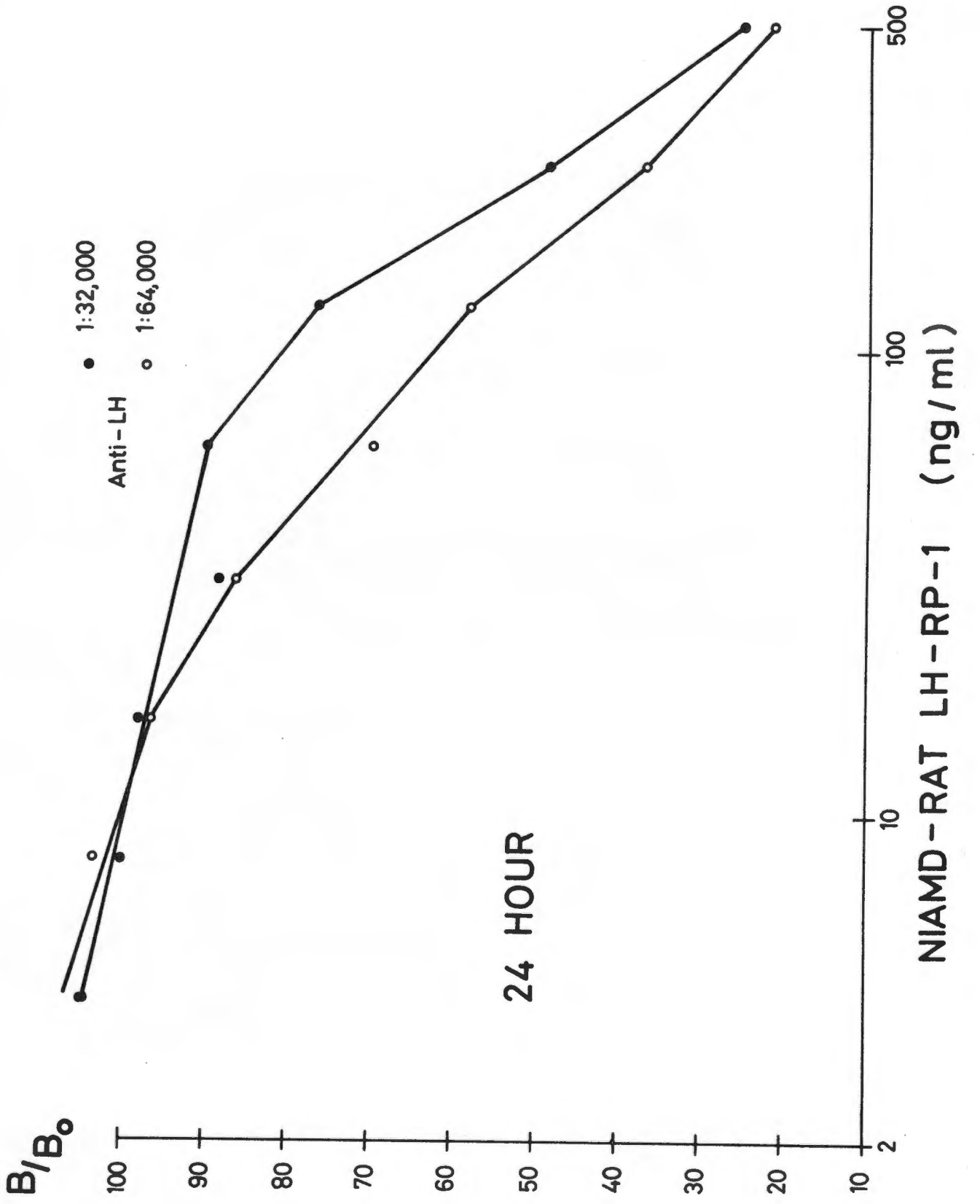


FIG 5 Duration of First Incubation Period: Standard curves obtained using first incubation period of 24 hours

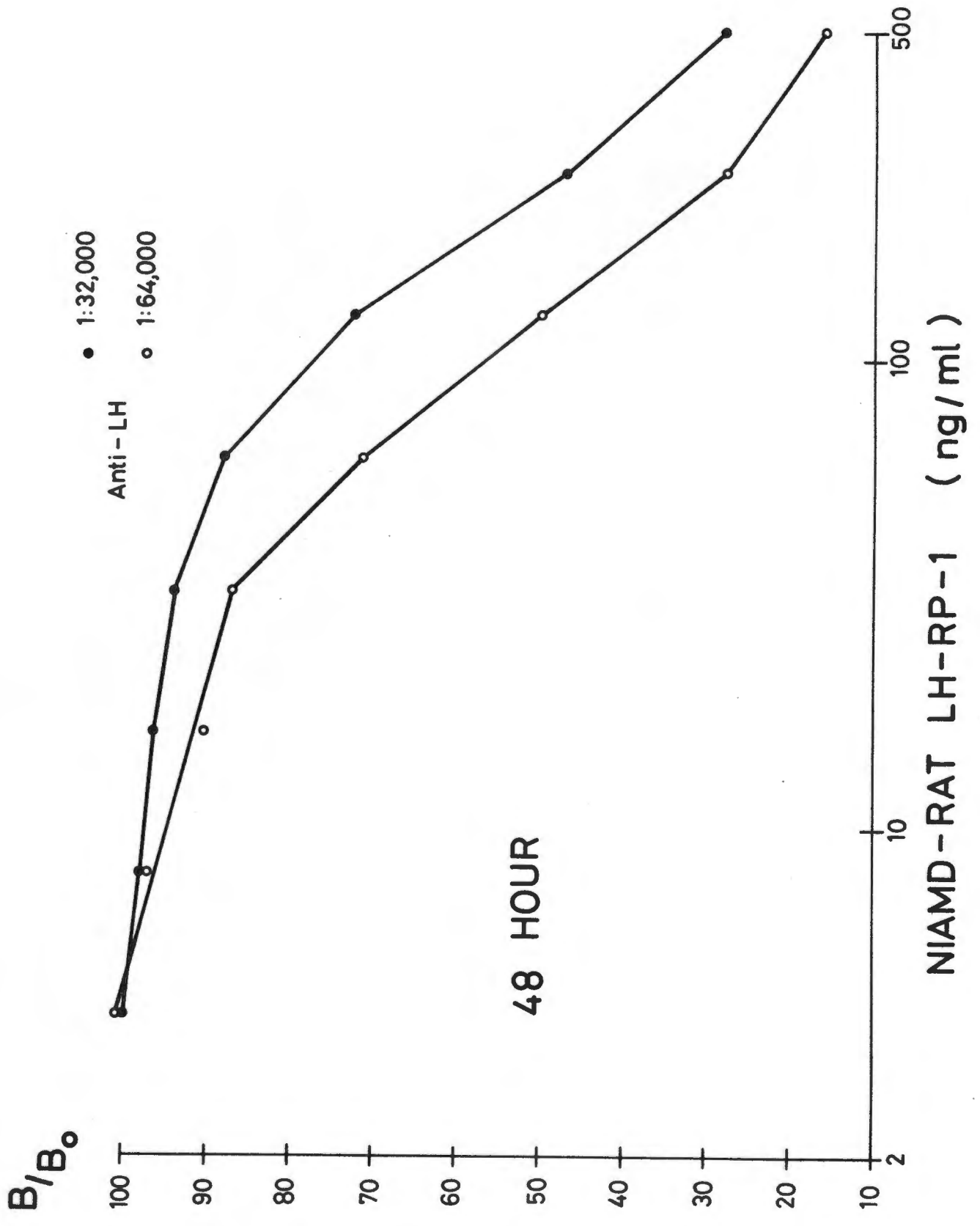


FIG 6 Duration of First Incubation Period: Standard curves obtained using first incubation period of 48 hours

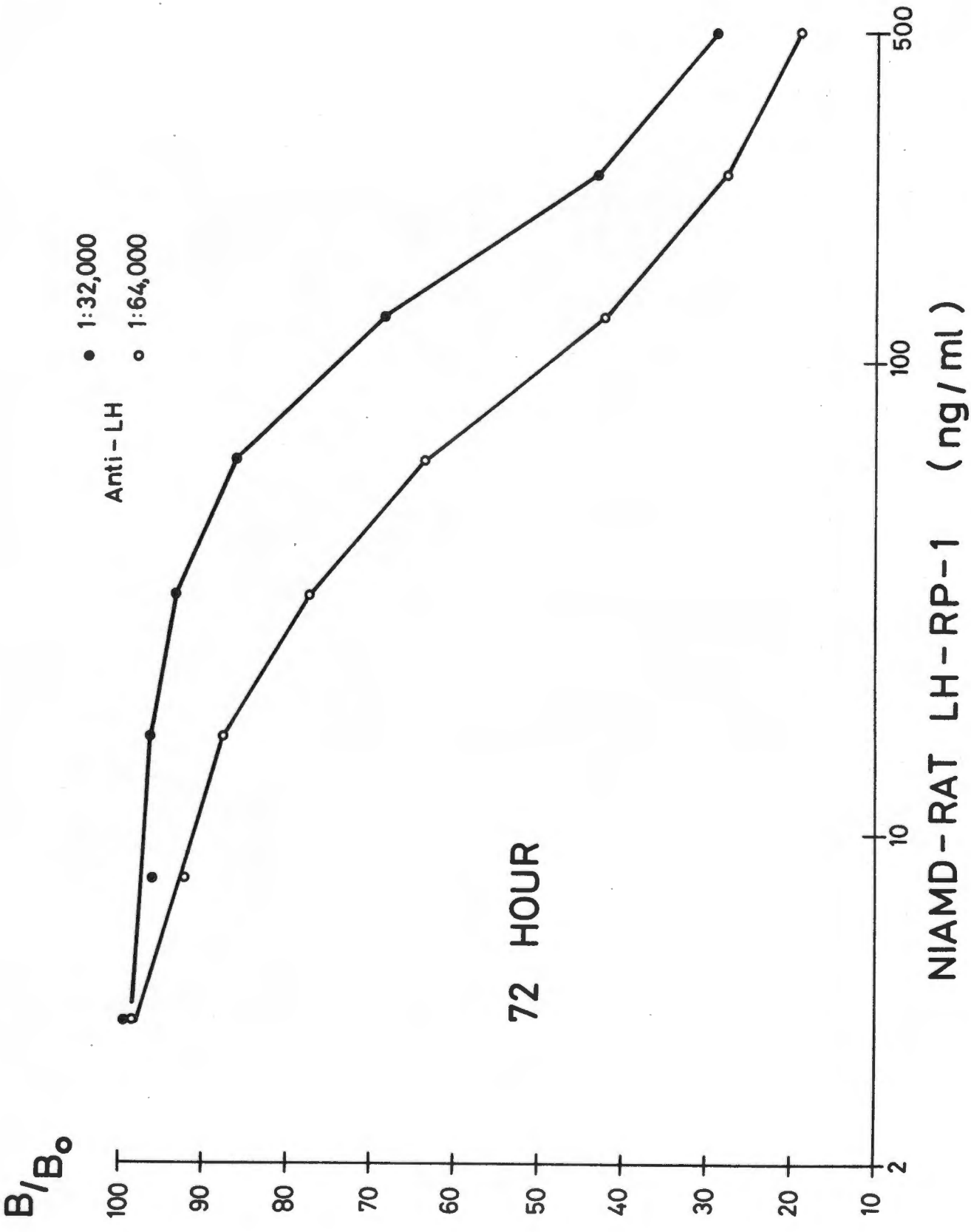


FIG 7 Duration of First Incubation Period: Standard curves obtained using first incubation period of 72 hours

Second Incubation Time

This study was performed in order to determine the optimum incubation period (between addition of labelled LH and addition of second antibody to the assay tubes) which would yield the highest percentage binding of labelled LH to the anti-LH.

Four sets of tubes, each set containing 3 different anti-LH dilutions, were arranged and filled with reaction components as shown in TABLE ....6. This study, in effect, consisted of antibody titrations coupled with varied second incubation time.

After addition of labelled LH (10,000cpm) to each tube, sets 1, 2, 3 and 4 were incubated at 4°C for 72, 96, 120 and 144 hours respectively, before addition of second antibody. The addition of the latter component, the subsequent 24 hour incubation and final centrifugation before counting were performed as described in the assay method (see page .56.) Results are depicted graphically in FIG ..8.. (App.TABLE ..5..) The curves indicate significantly greater binding of labelled LH to anti-rat LH after 144 hours incubation, as compared with the incubation times of shorter duration. This occurred at all anti-rat LH dilutions tested. In view of the earlier finding concerning a n optimum anti-rat LH dilution of 1/64,000 (see page ....60.), it appears that a second incubation time of 144 hours would prove most satisfactory under the assay conditions. The relatively low percentage of total counts bound to antibody in this study may be attributed to the following:

a) Antibody was diluted in a buffer containing 3% NRS - at

present, a buffer containing EDTA and 4% HSA is used, thus lowering NSB;

b) Labelled LH was diluted in a buffer containing no HSA - label is now diluted in a buffer containing 4% HSA.

c) Buffer was added to all the tubes to increase the volume of the reaction mixture. Appropriate buffer is at present added only to the non-specific and zero binding tubes which contain no first antibody and standard LH respectively.

TABLE 6

SECOND INCUBATION TIME

Tube	Buffer (A)*	Anti-LH in (B)* Volume	Dilution	Label in (A)	NRS & ARGG
TC	-	-	-	50µl	-
NSB	200µl	-	-	50µl	100µl
1-3	100µl	100µl	1:10 x 10^{-2}	50µl	100µl
4-6	100µl	100µl	1:80	50µl	100µl
7-9	100µl	100µl	1:640	50µl	100µl

* (A) = 0.01M PSB

* (B) = 0.01M PSB containing 3% NRS

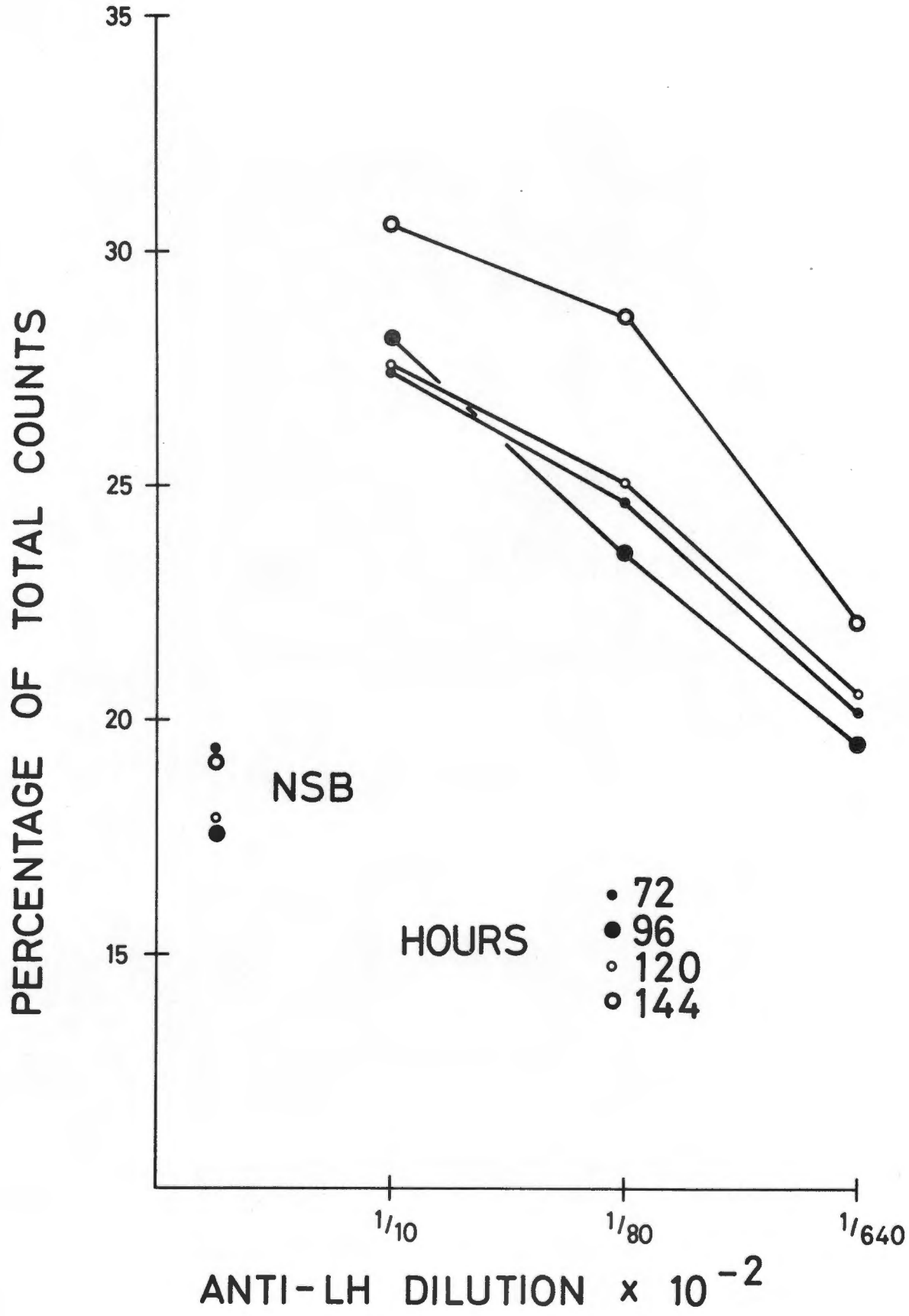


FIG 8 Duration of Second Incubation Period

R E L I A B I L I T Y C R I T E R I A

In order to define the limits of accuracy of the assay system and to quantify the magnitude of the errors inherent in the technique, studies were designed to investigate

1. a) the effects of storage on labelled rat LH;
   b) immunoreactivity of the buffer eluate from the CF 11 column;
2. immunological behaviour of unknown and standard LH;
3. reproducibility of the assay system;
4. sensitivity of the assay system;
5. the magnitude of errors involved in the use of different automatic pipetting devices.

-----oOo-----

1. a) Storage of labelled rat LH

The lability of the iodinated rat LH was tested by storing the crude label (derived from the first Sephadex peak) at -18°C for 5 days. Elution patterns off the cellulose CF 11 column, before and after storage, were compared and are shown in FIG .9... (see Appendix TABLE ....6) From these elution patterns it is apparent that the percentage of intact, labelled LH dropped from 96%+ before storage to 42%+ after storage, thus demonstrating approximately 50% breakdown of labelled hormone over the 5 day period. On the basis of these findings, fresh iodinated hormone was used for each assay.

b) Immunoreactivity of the buffer eluate from the CF 11 column

A standard curve was prepared in the usual manner, but the radioactive material obtained from the buffer eluate off

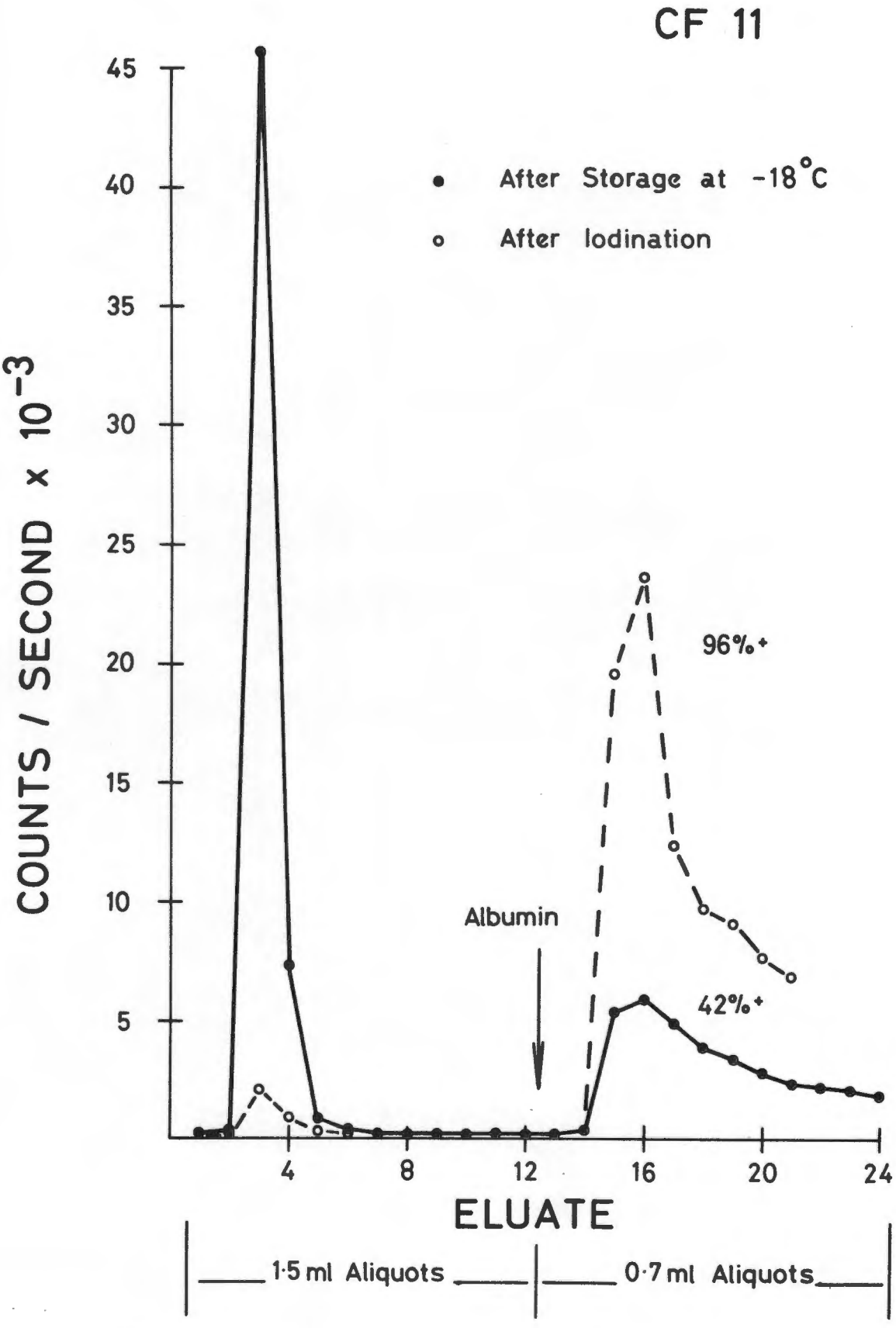


FIG 9 Cellulose CF 11 elution patterns immediately following iodination and after storage of labelled rat LH at -18°C for 5 days

the CF 11 column was used as label. This material is said to consist largely of labelled peptide fragments and free ^{125}I. Following suitable dilution with 0.01M PSB EDTA containing 4% HSA, this fraction was added to the tubes. The results, shown in Appendix TABLE ..7. confirm that

a) the radioactive material from the first CF 11 peak contains no intact, labelled hormone (at least not in sufficient concentration for purposes of running an assay; and

b) the antibody to rat LH binds very little free ^{125}I or breakdown products. In most cases the degree of binding did not exceed the value found in the non-specific binding tubes.

2. Immunological behaviour of unknown and standard LH (Parallelism)

In an attempt to verify comparable immunological behaviour between unknown sera and standard LH over a wide range of LH levels, doubling dilutions of two sera, A and B, varying between 1 in 2 to 1 in 16, were prepared in 0.01M PSB containing 4% HSA and assayed in the usual manner.

B/B_o values were plotted on the same graph as the standard curve and are shown in FIG ....10. (see Appendix TABLE ....8.)

In addition, the data depicted in this figure have been re-plotted in logarithmic form (FIG ....11.) following transformation of the B/B_o values to the logit notation (see Appendix TABLE ..9.. and statistical methods, page ....98.)

From the manner in which the dilutions of the sera parallel the standard curve, it is apparent that the sera behave immunologically in a similar manner to the standard LH.

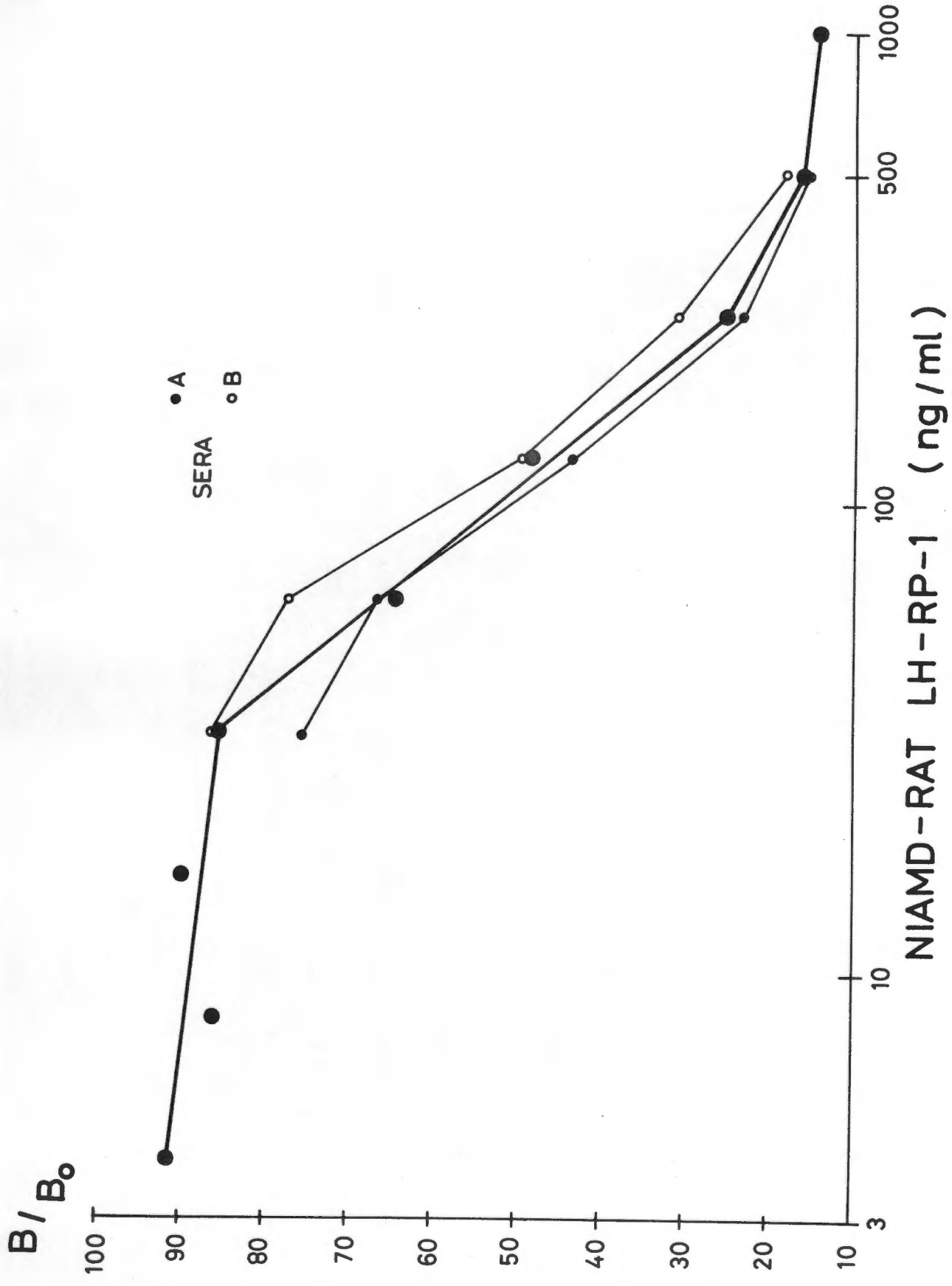


FIG 10 Demonstration of Parallelism between standard curve and unknown sera (A and B).

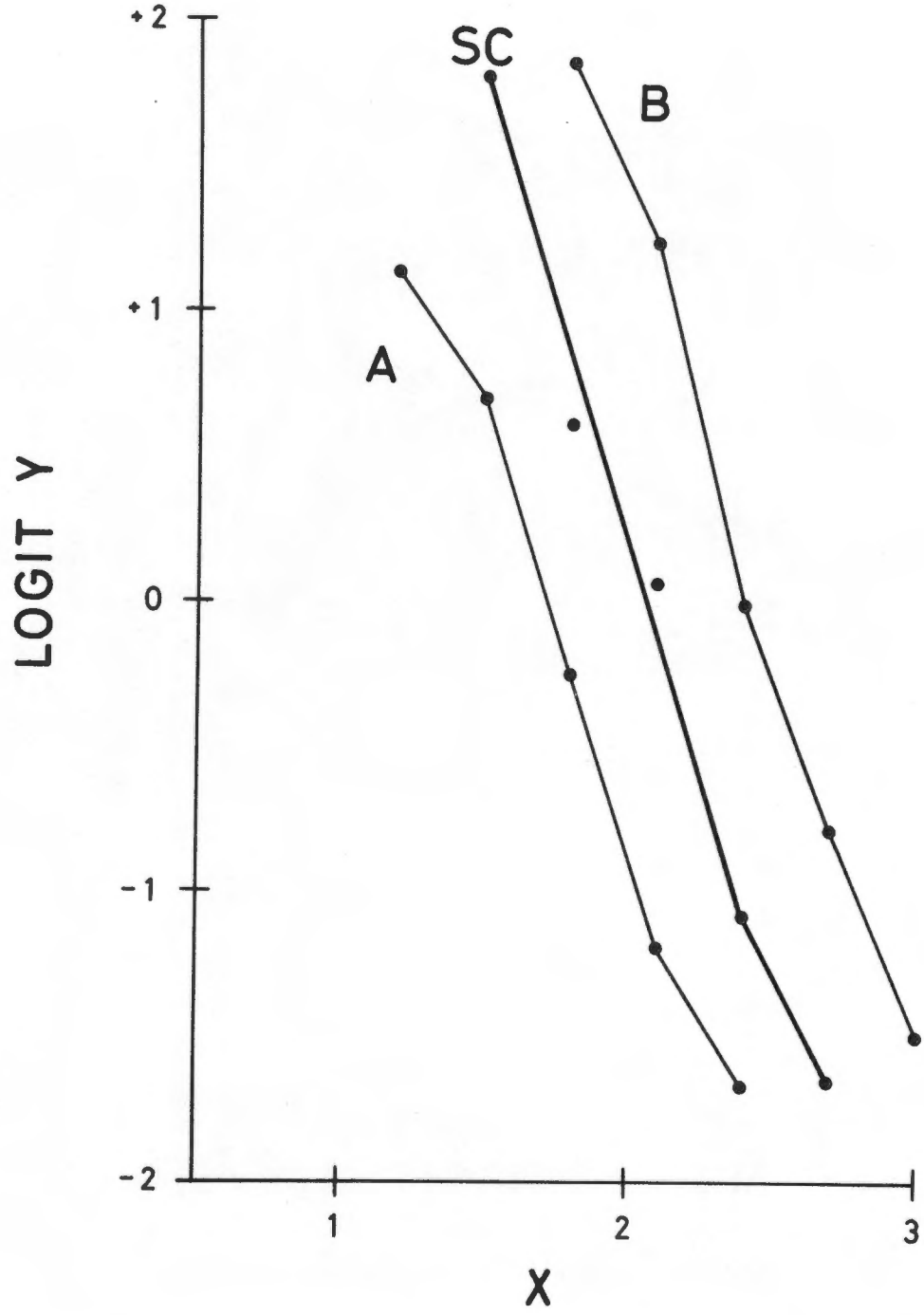


FIG 11 Demonstration of parallelism between standard curve (SC) and unknown sera (A and B), on a logit plot

3. Reproducibility of the assay system

The following studies were carried out in order to quantify the reproducibility of the assay technique - i.e. the limits within which B/B_o values, derived from both independent standard curves (inter-assay variation) and within a given standard curve (intra-assay variation), fall.

Inter-assay variation was quantified using B/B_o values derived from 5 consecutive standard curves. Mean B/B_o values and standard deviations at each point along the curve are shown in FIG .12. (see Appendix TABLE ..10) The standard deviations range between 2.0 and 5.4 but, when expressed as coefficients of variation (CV), the range increases from 2.7 to 11.9 Furthermore, the higher coefficients of variation apply to the greater concentrations of LH (250 and 500ng/ml) which fall along a relatively "flat" portion of the standard curve, while the lower values are associated with the steeper segment of the curve.

The mean coefficient of inter-assay variation, using all points along the standard curve, was found to be ±6.6%, as compared with ±7.5% (using pooled serum specimens) reported by the original workers (152). Similar use of pooled serum specimens in the present study has revealed a coefficient of variation of ±6.4% (see Appendix TABLE .10.)

Intra-assay variation was quantified by preparing a standard curve in the usual manner, but with 5 replications of those tubes containing 32, 62.5, 125 and 250 ngLH/ml. Mean B/B_o values and standard deviations at each of the four points are shown in FIG .13. (see Appendix TABLE .11.)

The mean coefficient of intra-assay variation over the steep

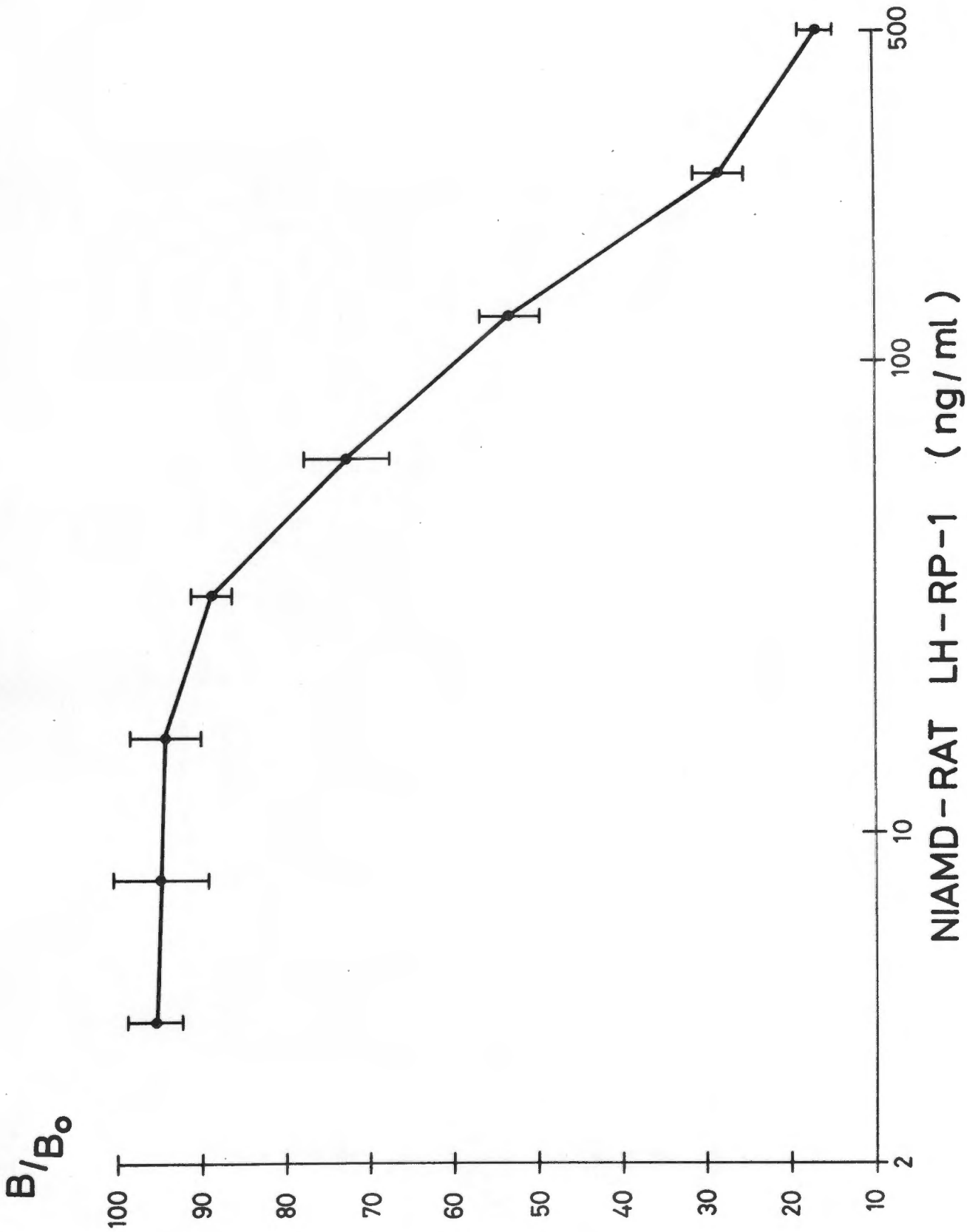


FIG 12 Inter-assay Variation: Mean B/B_o ($\pm$SD) at each point along the standard curve (n=5). Coefficient of inter-assay variation = $\pm$ 6.6%

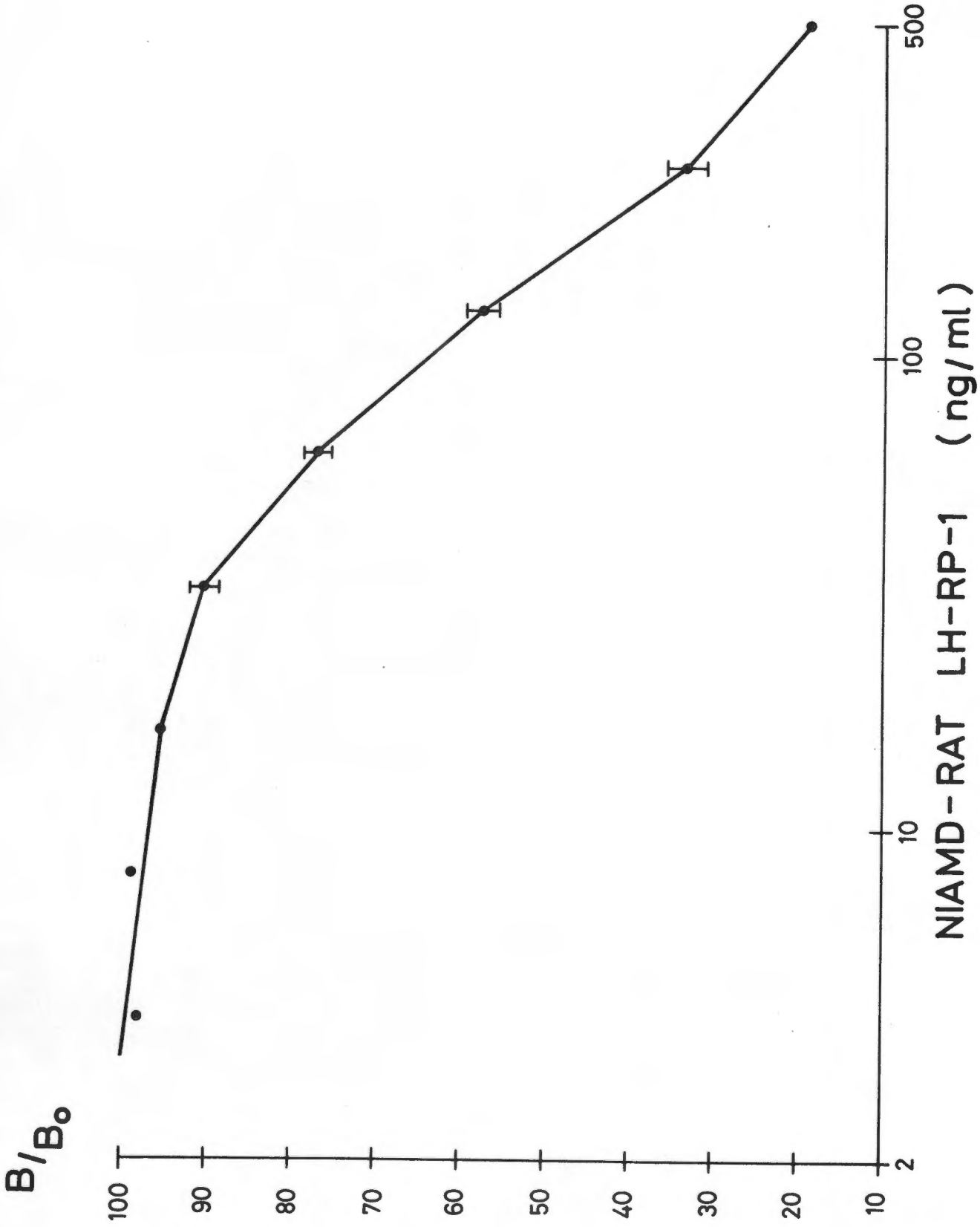


FIG 13 Intra-assay Variation: Mean B/B_o ($\pm$SD) at points along the standard curve corresponding to 32, 62.5, 125 and 250 ngLH/ml (n=5). Coefficient of intra-assay variation = $\pm$ 3.1%

portion of the curve was found to be ±3.1% as compared with ±5% (using pooled serum specimens) reported by the original workers (152)

4. Sensitivity of the assay system

The sensitivity of an assay is defined as the least amount of hormone which can be distinguished from no hormone (144).

According to this definition, the sensitivity of the present assay system is 16ng/ml LH, since the B/B_o value for this amount of LH was significantly lower than the zero-binding value ($P<0.05$) while the corresponding value for 32ng/ml LH was highly significantly different from zero ($P<0.001$) (see Appendix TABLE 10.)

Furthermore, the steep portion of the standard curve is most suitable for accurate interpolation of LH concentrations, and it is for this reason that unknown samples were diluted whenever LH concentrations greater than 250ng/ml were anticipated.

5. Errors involved in the use of different pipetting devices

A study was carried out in order to quantify the magnitude of the errors inherent in the use of each of the following pipetting devices:

a) Hamilton Syringe (50µl) : Hamilton Microlitre Syringe No. 705, Bonaduz.
b) Repette (2ml) : Jencons Scientific Equipment
c) Finnpipette (50-250µl) : Jencons Scientific Equipment

Each pipette was used to deliver 50µl of labelled rat LH (approximately 12,000cpm/50µl) into each of 20 tubes. The

radioactivity of each tube was measured for 1 minute. Mean counts/min, standard deviation and coefficient of variation for each pipetting series are shown in TABLE ..7.. Coefficients of variation (SD as percentage of mean) using the Hamilton Syringe, Repette and Finnpipette were found to be 0.69%, 2.37% and 1.46% respectively. The coefficients of variation for all 3 systems were within acceptable limits and for this reason the Repette was used for addition of first and second antibodies, buffer and label to the assay tubes, while the Finnpipette was used for preparation of doubling dilutions of LH standard and for the pipetting of unknown serum or plasma into the assay tubes.

TABLE ..7...

ERRORS INVOLVED IN THE USE OF DIFFERENT PIPETTING DEVICES

(Numbers below represent "Counts/min")

Tube	Hamilton	Repette	Finnpipette
1	13,656	12,340	13,980
2	13,453	13,222	13,949
3	13,806	12,373	14,071
4	13,657	12,621	13,719
5	13,688	12,589	13,809
6	13,623	12,672	13,747
7	13,674	12,762	13,739
8	13,754	13,049	13,869
9	13,799	12,490	13,460
10	13,704	12,922	13,644
11	13,824	13,077	14,159
12	13,742	12,963	13,822
13	13,717	13,104	13,513
14	13,703	13,243	13,924
15	13,711	12,873	13,772
16	13,689	13,155	13,396
17	13,832	13,343	13,618
18	13,569	13,042	13,526
19	13,602	13,372	13,574
20	13,547	13,098	13,644
Mean	13,688	12,916	13,747
SD	94.25	305.39	200.49
CV	0.69%	2.37%	1.46%

Where SD = standard deviation

CV = coefficient of variation

C H A P T E R 3

E X P E R I M E N T A L M E T H O D S

EXPERIMENTAL METHODS

(1) EXPERIMENTAL ANIMALS

Throughout these studies adult male or female rats of the Wistar-strain were used. The animals were housed in a windowless, air-conditioned room with ambient temperature 20 to 24°C and given free access to rat chow (Epol rat cubes - Vereeniging Consolidated Mills Ltd) and water at all times. Lighting of the animal room was provided by two 120cm Phillips 40W fluorescent tubes, and the light/dark pattern regulated by a Venerette automatic light switch such that the animals received 14 hours of light (6am to 8pm) and 10 hours of darkness per day.

(2) ADMINISTRATION OF ANAESTHETICS

The various anaesthetics used in these studies, together with the dose and route of administration of each, were as follows:

Parenteral administration (intraperitoneal injection)

Urethane (Ethyl Carbamate) - British Drug Houses

1.2g/Kg + 0.4g/Kg supplementation as required.

Inactin - Promonta

100mg/Kg + 17mg/Kg supplementation as required.

Sagatal (Pentobarbitone Sodium) - Maybaker (SA) (PTY) Ltd (60mg/ml soln)

50mg/Kg + 25mg/Kg supplementation as required.

Sodium Pentobarbitone (Powder) - Petersen Ltd (50mg/ml water)

50mg/Kg + 5mg/Kg supplementation as required.

Inhalation anaesthesia

Trilene (trichloroethylene) - ICI Ltd.

Used for induction of anaesthesia only.

Ether (diethyl) - Natal Cane By-Products Ltd.

For maintenance of anaesthesia following trilene induction.

(3) ADMINISTRATION OF DRUGS

Heparin (Evans Medical Ltd.)
5,000 Units/ml.

Where necessary, animals were heparinized by single intravenous injection of either

a) 100 Units heparin/0.2ml saline - for prolonged heparin therapy (e.g. audiostimulation studies); or

b) 500 Units heparin/0.2ml saline - for acute studies (e.g. LRH studies).

Terramycin (20mg/ml) - Pfizer Laboratories

Chronically cannulated animals received 10mg terramycin (0.5ml) subcutaneously following surgery.

LRH - Ayerst Laboratories

All doses were administered intravenously in 0.2ml saline.

(4) SURGICAL TECHNIQUES

a) Bilateral ovariectomy: Following induction of anaesthesia with trilene and maintenance with ether, the hair covering the operative areas was clipped with an electric clipper and the skin sterilized with a 1:4 solution of tincture of iodine in 70% ethanol.

A longitudinal skin incision (± 5mm) was made on either side of the body at a point 1 cm caudal to the lower margin of the rib cage and 1cm lateral to the vertebral column. Incision of the underlying posterior abdominal musculature enabled the ovary and attached portion of the uterine horn to be drawn out through the wound with the aid of blunt forceps. The ovarian artery and vein, together with ± 5mms of uterine horn, were ligated with 2-0 silk and the ovary and attached portio n of the uterus excised. The ligated tissue was returned to the abdominal cavity and the incision in the abdominal wall closed with a single 2-0 silk suture. One autoclip sufficed to approximate the margins of the skin incision.
(All surgical instruments were sterilized by boiling for 20 minutes and the silk was stored in 70% ethanol. No antibiotics were administered to the animals.)
"Sham Ovariectomy" - the surgical procedure followed was similar to that for the ovariectomized rats, with the exceptions that

1) the ligature around the uterine horn and ovarian blood vessels was tied loosely; and
2) the ovary was not excised.

b) Cannulation of the Carotid Artery: The rats were anaesthetized by intraperitoneal injection of the particular anaesthetic selected. Following induction of anaesthesia (the depth of anaesthesia being gauged from the ratio thoracic/ abdominal respiration and the extent of abdominal muscle contraction in response to tail pinching) the ventral surface of the neck was shaved with an electric clipper and the animal pinned to a cork mat, in the supine position, via strips of

adhesive tape attached to the limbs. Extension of the neck by means of a pad of tissue greatly facilitated the following surgical procedure:

A midline skin incision permitted insertion of a polythene tracheotomy tube (Intramedic Polythene Tubing PE 120 - Clay Adams) followed by cannulation of the left common carotid artery using polythene tubing (Intramedic Polythene Tubing PE 360 - Clay Adams) which had been drawn out to the desired width over a flame. Care was taken to ensure that the Vagus nerve was not damaged.

The free end of the cannula was attached to a 1ml disposable syringe containing heparinized saline (2Units/ml), via a metal Luer needle attachment, the shaft of which was introduced into the flared end of the cannula and tied securely in place. The cannula was then advanced 2-3cms towards the heart and ligatured in position. Following arterial cannulation, the skin was sutured to minimize loss of body fluids.

c) <u>Chronic Jugular Cannulation:</u> The method selected for these studies consisted of a modification of the original technique of Steffens (203). Basically, the proqedure involves the placing of an indwelling jugular cannula, the free end of which is readily accessible for the sampling of central venous blood at any desired intervals of time.

Whereas the original method involved the use of a cannula which was firmly cemented to the skull of the animal, the technique was modified such that

1) it did not necessitate trephining of the skull;
2) it was simpler to perform;
3) it provided the worker with a flexible cannula, the free end of which was easily handled
    a) by the worker alone; and
    b) without requiring that the animal kept

its head still;

4) the free end of the cannula was beyond the normal reach of the animal's paws.

MATERIALS

22 gauge disposable syringe needle shaft (1.25cm length) bent as depicted in FIG ..14.

10cm length of silastic tubing (DowCorning 602-131 - ID .020" OD .037", Dow Corning Corporation.

Sterile saline for injection (M.L. Laboratories)

Heparin for injection (Evans Medical Ltd)

Polyvinylpyrrolidone (PVP) (E. Merck AG)

1gm in 2ml heparinized saline (500 Units/ml).

Sodium Pentobarbitone (Courtesy of Petersen Ltd) 50mg/ml water

30cm polythene tubing (Intramedic Polythene Tubing, Clay Adams Division of Becton, Dickinson & Co.)
PE 60 - ID 0.030" OD 0.048"
PE 360 - ID 0.148" OD 0.190"

22 gauge metal Luer needle attachment with 1cm length of smoothly polished shaft.

1/4 solution of tincture of iodine in 70% ethanol.

Terramycin solution (20mg/ml)

Surgical Instruments

M E T H O D

With the following exceptions, the method is identical with that of Steffens (203):

a) 250-350gm Wistar rats were used in these studies (Steffens used Sprague-Dawley rats);

b) Rats were anaesthetized by intraperitoneal injection of sodium pentobarbitone (50mg/Kg) (Steffens administered the

anaesthetic subcutaneously)

c) Prior to surgery, the hair covering the operative areas was clipped with an electric clipper and then shaved using a scalpel blade. (Steffens plucked the hairs with tweezers):

d) The skin was sterilized with a 1/4 solution of tincture of iodine in 70% ethanol. (Steffens used Chlorhexidine)

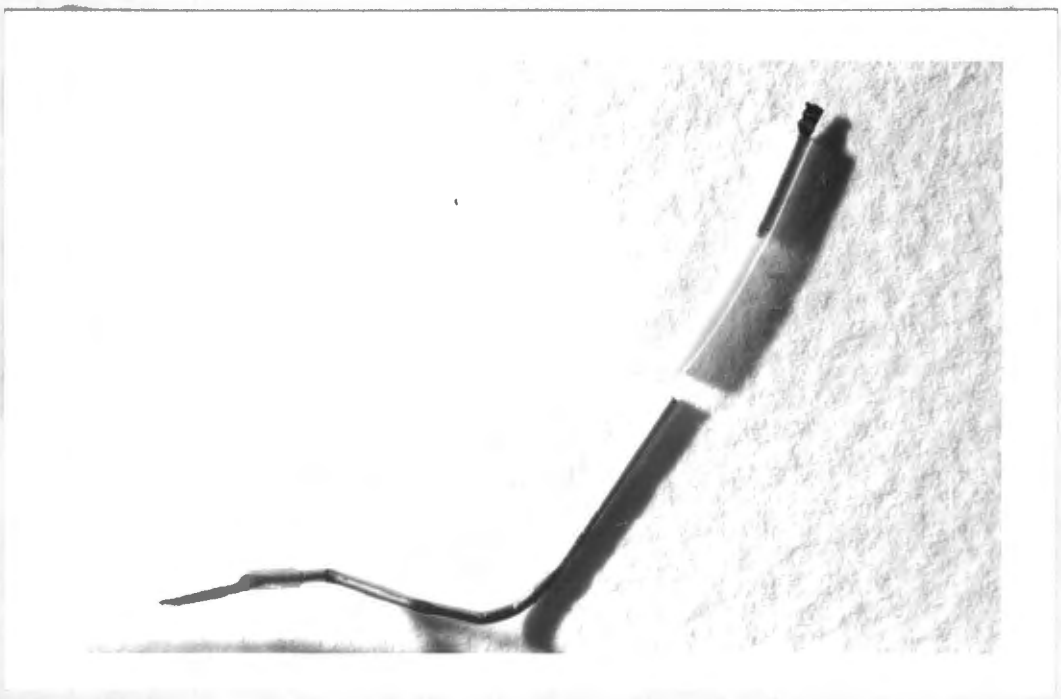
e) The right external jugular vein was pierced and cannulated at a point 5mm ~~rostral~~ caudal to the junction of the anterior jugular, acromiodeltoid and cephalic veins. (Steffens inserted the cannula into the wall of the thickest of these confluent veins at a point 2mm ~~caudal~~ rostral to their junction)

f) The depth of insertion of the cannula into the vein was not found to be critical for maintenance of cannula patency. (Steffens states that the cannula will remain patent only if inserted into the vein to a depth of 42mm, with respect to the point of insertion.) In the present studies the cannula was introduced into the vein and advanced a distance of about 3cms. The cannula was then either advanced or retracted until blood could be freely aspirated using a syringe attached, via polythene PE 60 tubing, to the free end of the cannula. Suitably placed ligatures were then tied to anchor the cannula in this position.

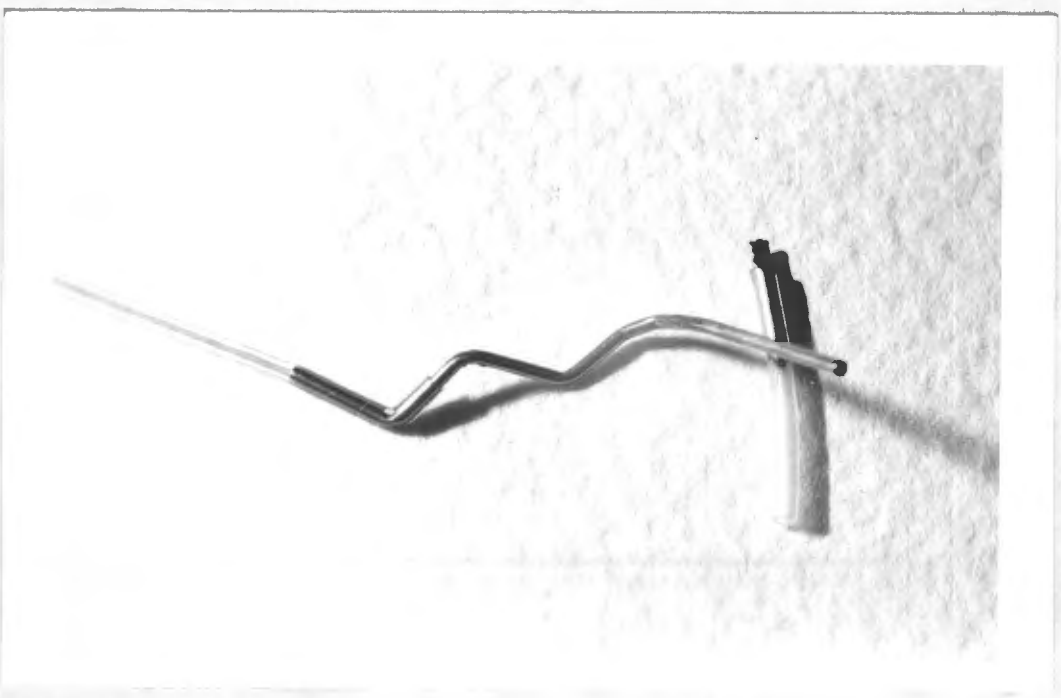
g) The free end of the cannula was not cemented to the skull, but was slipped over the end of the bent cannula needle as shown in FIG ..14.. This bent needle was located subcutaneously in the midline of the back such that

1) the silastic cannula passed down the right side of the neck; and

2) the free end of the cannula needle projected vertically from the back to a height of approx. 1cm.



(A)



(B)

FIG 14

Lateral (A) and dorsal (B) views of the bent cannula needle with polythene plug

h) The cannula needle was sutured in position, using 3-0 silk, with care taken to ensure that the knots of the sutures were located subcutaneously, thus reducing the possibility of them loosening with time.

i) Following cannulation of the vein, the wounds were swabbed out with terramycin solution and the animal given 10mg terramycin subcutaneously. (Steffens administered penicillin to his animals.)

j) Every two days the cannulae were flushed out with ± 1ml of saline and refilled with fresh PVP-heparin solution as described by Steffens.

The total duration of the operation varied between 20 and 40 minutes and the animals generally recovered within a day or two. In intact female rats, regular oestrous cycles were resumed 2-5 days after the operation, thus suggesting the occurrence of only a transient stress effect. Before drawing any blood samples, the animals were allowed a recovery period of at least 4 days following surgery.

On several occasions it was necessary to resuture the cannula needle. This was performed under light ether anaesthesia, following trilene induction, and the animals given a rest period of 3 days before being used in an experiment.

(5) BLOOD COLLECTION TECHNIQUES

Intact, non-cannulated animals - These animals were sacrificed by cervical dislocation and severance of the right external jugular vein and right common carotid artery, and a single blood sample collected.

Anaesthetized, acutely-cannulated animals

e.g. carotid cannula

Blood was drawn from the anaesthetized animal at 10 minute intervals using 1ml disposable syringes as follows:
30 secs prior to sampling blood, the plunger of the heparinized saline syringe, attached to the cannula, was withdrawn until arterial blood entered the tip of the syringe;
15 secs prior to sampling blood, the syringe was uncoupled from the Luer attachment and 1 drop of the blood-saline mixture allowed to fall on to a tissue, thus ensuring that the timed blood sample consist entirely of undiluted blood;
5 secs prior to sampling blood, a clean, dry 1ml syringe was coupled to the Luer attachment and the desired volume of blood aspirated.

Immediately following aspiration of the blood sample, heparinized saline was injected through the cannula. Care was taken to ensure that no more than 0.1 ml of saline was injected to accomplish this.

Conscious, unstressed, chronically cannulated animals

Blood samples were aspirated from these animals, via a length of polythene tubing (Clay adams PE 60, or PE 360 drawn out over a flame) slipped over the free end of the cannula needle, using either

a) disposable 1ml syringes. Heparinization of the animal was not essential, provided the cannula was flushed out with saline between drawing blood samples and refilled with PVP-heparin solution at the end of the sampling session; or

b) a peristaltic pump (Scientific Manufacturing Co.). Animals were heparinized by intravenous injection of

either 100 or 500 Units of heparin in 0.2ml saline.

Treatment of Blood Samples

All blood samples were allowed to clot at room temperature for at least 1 hour before being centrifuged at 3,000rpm for 10 minutes. The sera (or plasma samples) were stored at -18°C while awaiting LH radioimmunoassay.

(6) AUDIOSTIMULATION APPARATUS

The animals were exposed to the audiostimulus in a windowless, air-conditioned room (14 feet square and with height 8.5 feet) illuminated by four 120cm Phillips 40W fluorescent tubes, and with the light/dark pattern controlled as described on page ....87. Room temperature varied between 20.1 (Min) and 22.3 (Max) °C.

In all of the sound studies an intermittent sound stimulus was used, consisting of 20secs "on" and 40secs "off" throughout the stimulation period, the latter extending between 8pm and 6am, and again between 8am and 10am. (This stimulation period was controlled by an "Electro Boy" clock.)
The sound stimulus was generated by a sine wave audiogenerator (Model TE-22) connected via an amplifier to a 6 inch Coral speaker, the latter mounted in a cabinet and positioned 10ft 11ins away from the animal cages on the opposite side of the room. Frequency and/or intensity of the sound stimulus were varied according to the requirements of the particular study in progress. Intensity of the stimulus was continually checked with the aid of a sound level meter (Type No. 1551-A, General Radio Co., Mass., USA).

Precautions taken:

1) The animal cages were placed in exactly the same positions throughout all the studies;
2) The sound level meter was always placed in the same position before either checking or resetting sound intensity;
3) The same person (alone in the room) checked or reset the sound intensity each time this was done;
4) Frequent recordings of background "noise" were made;
5) No sound adjustments were made with animals in the room.

(7) MISCELLANEOUS TECHNIQUES

Haematocrit of blood from each male rat was estimated using heparinized capillary tubes, in a Hawksley Micro-haematocrit Centrifuge and Reader (Hawksley & Sons Ltd., England).

Blood Haemoglobin Concentration was estimated using a Spencer Haemoglobinometer.

Vaginal Smears were stained according to a modification of the method of Papanicolaou (164), and the animals ascribed to various stages of the oestrous cycle according to the relative proportions of epithelial cells, leucocytes and keratin present. (See Appendix TABLE .12. for method.)

(8) STATISTICAL METHODS

The methods of statistical analysis employed in the interpretation of results included the following:

Measures of central tendency and of dispersion (202)

a) Arithmetic Mean $(\bar{x}) = \frac{\Sigma x}{n}$

b) Standard Deviation (SD) $= \sqrt{\frac{\Sigma (x-\bar{x})^2}{n-1}}$ (for small samples)

c) Coefficient of Variation (CV) $= \frac{SD}{Mean} \times 100$

Comparison of small samples using 2-tail Student's "t"-test (202)

a) Comparison of 2 different samples:

$$t = \frac{\bar{x} - \bar{y}}{\sqrt{\frac{n_1 S_1^2 + n_2 S_2^2}{n_1 + n_2 - 2} \left(\frac{1}{n_1} + \frac{1}{n_2} \right)}}$$

b) Same sample, before and after "treatment". Paired t-test.

$$t = \frac{\bar{d} - 0}{\sqrt{\frac{\Sigma (d - \bar{d}^2)}{n(n-1)}}}$$

Logit Notation (144)

$$\text{Logit } Y = \log_e (Y/1-Y)$$

CHAPTER 4

EXPERIMENTAL RESULTS

S E C T I O N 1

THE MEASUREMENT OF BASAL LH SECRETION IN ADULT MALE AND FEMALE RATS

Since basal levels of LH secretion represent an essential prerequisite to the logical interpretation of experimental data, particular attention has been devoted to the estimation of resting serum (or plasma) LH concentrations in normal male, intact female and long-term bilaterally ovariectomized rats. The respective studies were conducted with the following objectives in mind:

a) To quantify the magnitudes of LH secretion in both male and intact female rats in the absence of intentional exteroceptive influences; and to compare plasma LH concentrations in the conscious animal with those in comparable rats sacrificed by cervical dislocation;

b) To study fluctuations in serum LH concentrations during the normal oestrous cycle of the rat;

c) To study the time course of the LH-secretory response to bilateral ovariectomy; and

d) To investigate the pulsatile pattern of basal LH secretion in long-term bilaterally ovariectomized rats.

---oOo---

a) BASAL LH SECRETION IN MALE AND FEMALE RATS

Males Blood was sampled continuously by peristaltic pump for 1 hour from 12 chronically cannulated adult rats. In addition, 9 adult rats were sacrificed by cervical dislocation and a single blood sample collected from each animal. Plasma LH levels, shown in TABLE .8. (see Appendix Tables .13. and .14.) in the conscious, unstressed group of rats

TABLE 8

BASAL LEVELS OF SERUM LH IN MALE, INTACT FEMALE AND LONG-TERM BILATERALLY OVARIECTOMIZED RATS

	Conscious			Cervical Dislocation				
	n	$\bar{x}$ ng/ml	SD	n	$\bar{x}$ ng/ml	SD	t	P<
Males	72	46.8	27.2	9	104.4	35.9	5.6857	0.001
Ovariectomized females	15	701.7	65.8	14	699.6	120.3	0.0568	0.975
Intact females	12	77.9	22.9	9	103.9	28.6	2.1995	0.05

(46.8 ± 27.2 ng/ml) were significantly lower ($P < 0.001$) than in the group sacrificed by cervical dislocation (104.4 ± 35.9 ng/ml).

Intact Females Three chronically cannulated adult females and 9 intact (non-cannulated) females were used in this study. Vaginal smears were examined daily, and when each rat had completed two regular (4-day) oestrous cycles, blood samples (0.6ml) were aspirated from the cannulated animals on both the dioestrous and metoestrous days of 2 consecutive cycles. Blood samples were also collected from 9 intact animals which were sacrificed by cervical dislocation during the same stages of the reproductive cycle. Results are depicted in TABLE .8. (see Appendix Table .15.). As in the case of the male rats, plasma LH concentrations in the conscious, unstressed animals (77.9 ± 22.9 ng/ml) were significantly lower ($P < 0.05$) than in the group sacrificed by cervical dislocation (103.9 ± 28.6 ng/ml)

Bilaterally Ovariectomized Rats Circulating LH concentrations in conscious, unstressed animals were compared with those in animals sacrificed by cervical dislocation. The former group consisted of 6 adult, chronically cannulated females which had been ovariectomized 1 month earlier. Blood was sampled continuously by peristaltic pump for 1 hour from each animal. The procedure was repeated at 4 to 6 day intervals. The latter group consisted of 14 ovariectomized rats which were sacrificed by cervical dislocation 2 to 3 weeks after operation, and a single blood sample collected from each. Results are depicted in TABLE .8.. (see Appendix Table .16.). Plasma LH concentrations in the group of conscious animals (701.7 ± 65.8 ng/ml) were not significantly different from

levels in rats sacrificed by cervical dislocation (699.6 ± 120.3 ng/ml).

b). FLUCTUATIONS IN SERUM LH LEVEL DURING THE NORMAL OESTROUS CYCLE OF THE RAT

Three adult female rats, weighing between 250 and 300 grams, were anaesthetized and indwelling jugular cannulae placed as described on page 90. Vaginal smears were examined daily thereafter. Once each animal had completed two regular (4-day) oestrous cycles, 0.6ml blood samples were aspirated at 4pm on each day of 2 consecutive cycles and the sera assayed for LH. (The appearance of vaginal smears on each of the blood sampling days is depicted in Appendix Table 17.).

The mean dioestrous serum LH concentration was 78.2 ± 29.3 ng/ml, this value increasing 4-fold to reach a prooestrous level of 345.3 ± 224.8 ng/ml ($P < 0.025$). At oestrus, the serum LH level had decreased to 74% of the pro-oestrus value (256.7 ± 79.8 ng/ml), although still remaining highly significantly elevated above the dioestrous concentration ($P < 0.001$), while at metoestrus serum LH concentration had dropped to 77.5 ± 17.3 ng/ml, which was not significantly different from that at dioestrus (FIG 15. Appendix Table 18.). Although the mean LH concentration at oestrus was lower than that at pro-oestrus (257 ng/ml and 345 ng/ml), the difference between the two was not statistically significant.

c) EFFECT OF BILATERAL OVARIECTOMY ON SERUM LH LEVEL

The aim of this investigation was to measure serum LH concentrations at specific time intervals following bilateral

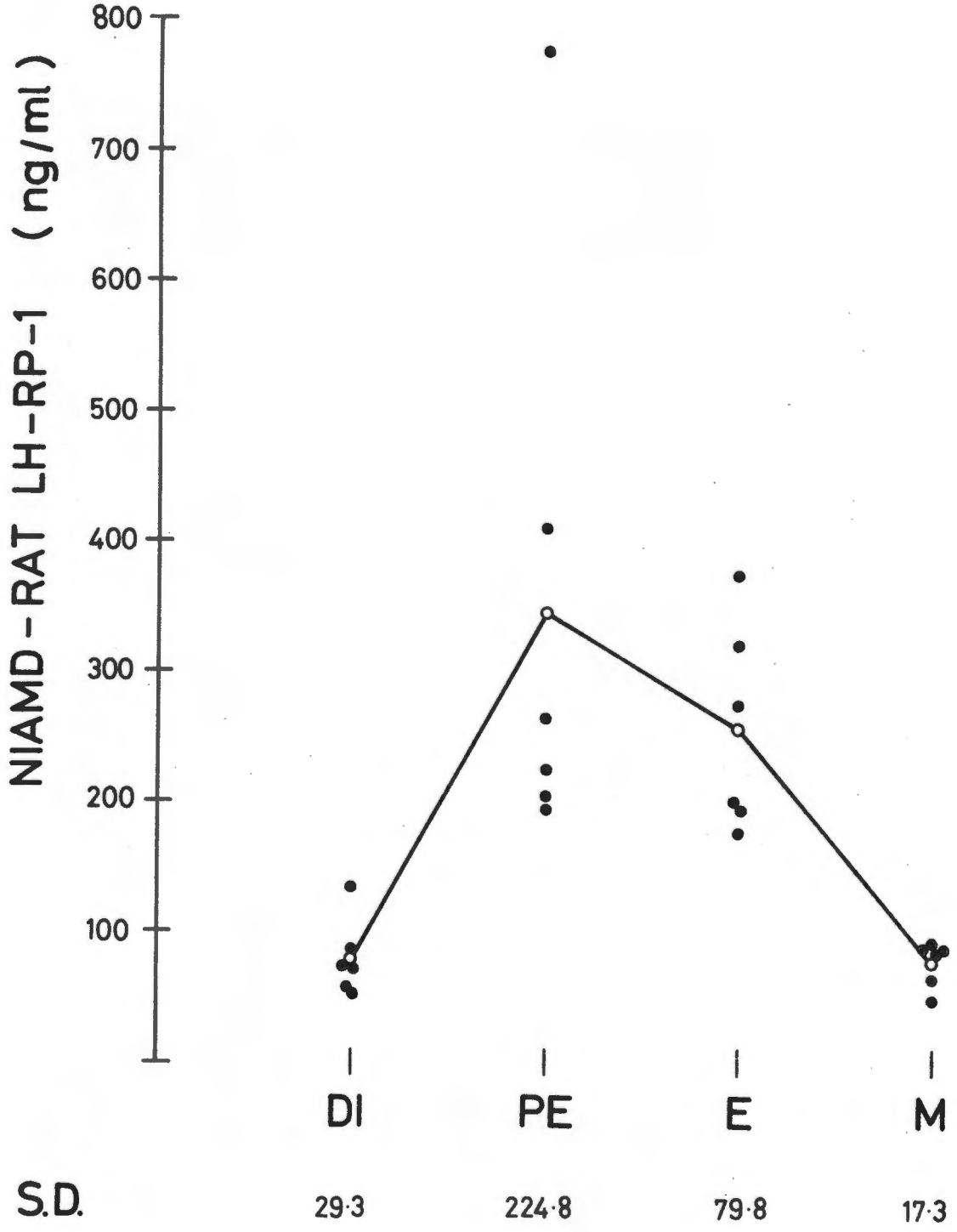


FIG 15 Mean serum LH concentrations during the normal rat oestrous cycle. DI, PE, E and M refer to the dioestrous, pro-oestrous, oestrous and metoestrous stages of the cycle. Individual concentrations represented by the filled circles

ovariectomy.

Eighteen adult female rats, weighing between 250 and 300 grams, were bilaterally ovariectomized as described on page 88. In addition, 5 sham-operated and 5 intact animals represented controls for the study. The experimental animals were sacrificed by cervical dislocation in groups on days 7, 12, 17 and 21 following operation, and a single blood sample collected from each. The sham-operated and intact females were sacrificed during the dioestrous stage of the reproductive cycle and approximately 7 days after "operation".

Serum LH concentrations in intact dioestrous rats (A), in sham-operated rats (B) and in animals sacrificed on days 7, 12, 17 and 21 following bilateral ovariectomy are shown graphically in FIG 16 (see Appendix Table 19). Mean serum LH concentration in sham-operated control animals was 90.5 ± 26.2 ng/ml, while that in the intact dioestrous animals was slightly higher - 120.4 ± 24.7 ng/ml - but not significantly different. Seven days after operation a 4-fold increase in serum LH concentration had occurred ($P < 0.005$). This value increased further by day 12 to 702.5 ng/ml which was 7 times greater than that in the sham-operated animals ($P < 0.001$). Thereafter, serum LH concentration remained relatively constant, showing only a minimal decline between days 17 and 21 following ovariectomy.

No statistically significant difference was demonstrated between serum LH levels on days 7 and 12 following operation ($P < 0.1$).

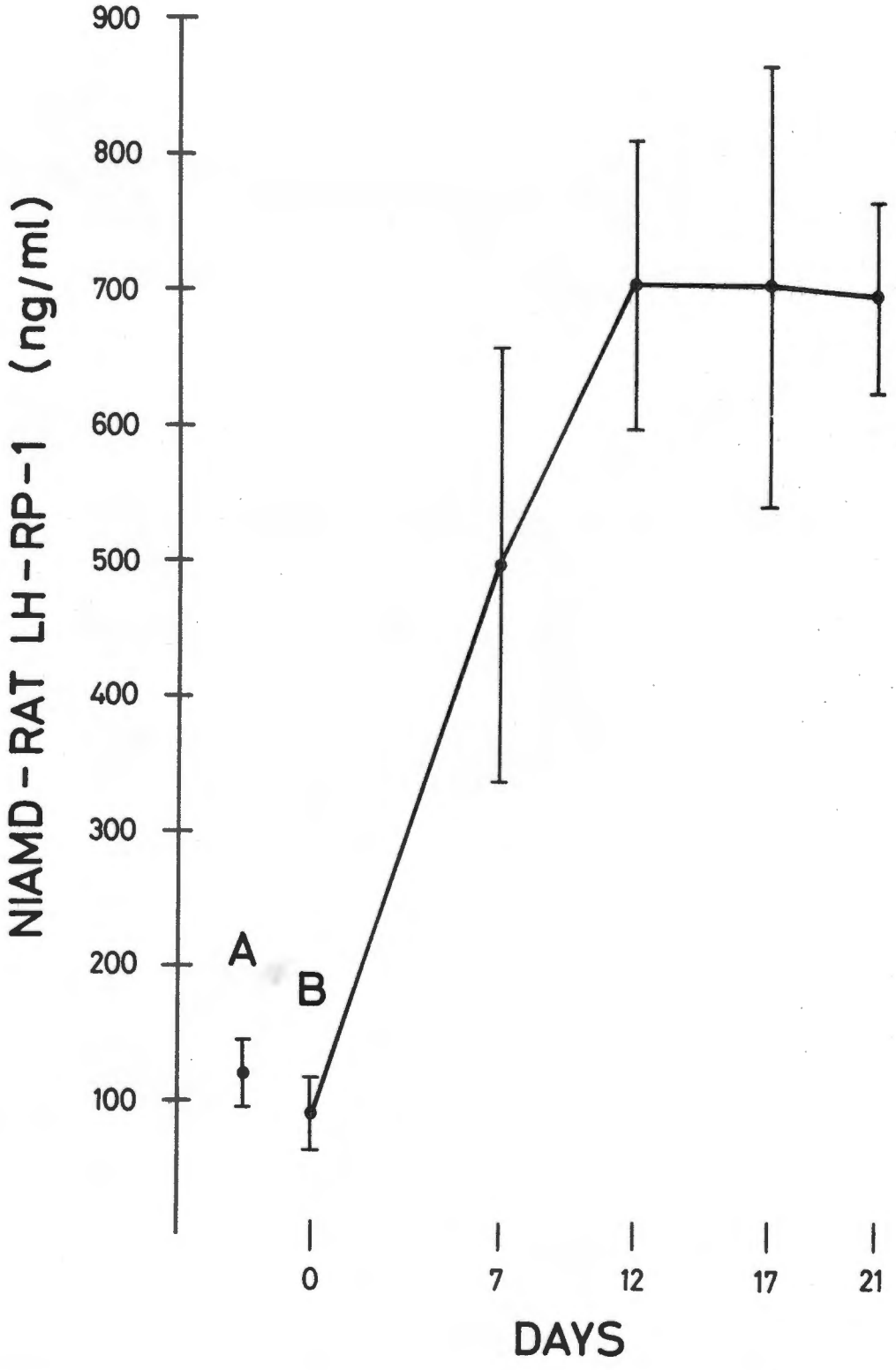


FIG 16 Mean serum LH concentrations (±SD) in intact (A) and sham-operated (B) dioestrous rats, and in animals sacrificed on days 7, 12, 17 and 21 following bilateral ovariectomy

d) PATTERN OF BASAL LH SECRETION IN LONG-TERM BILATERALLY OVARIECTOMIZED RATS

Three adult female rats (±300 grams), which had been bilaterally ovariectomized 5 months earlier, were cannulated as described on page 90. Following a recovery period, each animal received an intravenous injection of 500 Units of heparin in 0.2ml saline. One hour later blood was sampled simultaneously from each rat, by peristaltic pump, for 70 minutes (flow rate 4.2 ml/hour). Five-minute fractions of blood (0.35ml) were collected, and the plasma assayed for LH.

Changes in plasma LH concentration with time are shown in FIGS 17 18 and 19 (see Appendix Table 20.). Plasma LH level fluctuated in a pulsatile manner between the limits of 475 and 800 ng/ml, with mean values in rats I, II and III of 635.4 ± 103.4, 682.5 ± 98.2 and 692.9 ± 110.7 ng/ml respectively. In addition, the time interval between the pulses of LH secretion varied between 10 and 25 minutes (Mean = 16.7 ± 5.5 minutes).

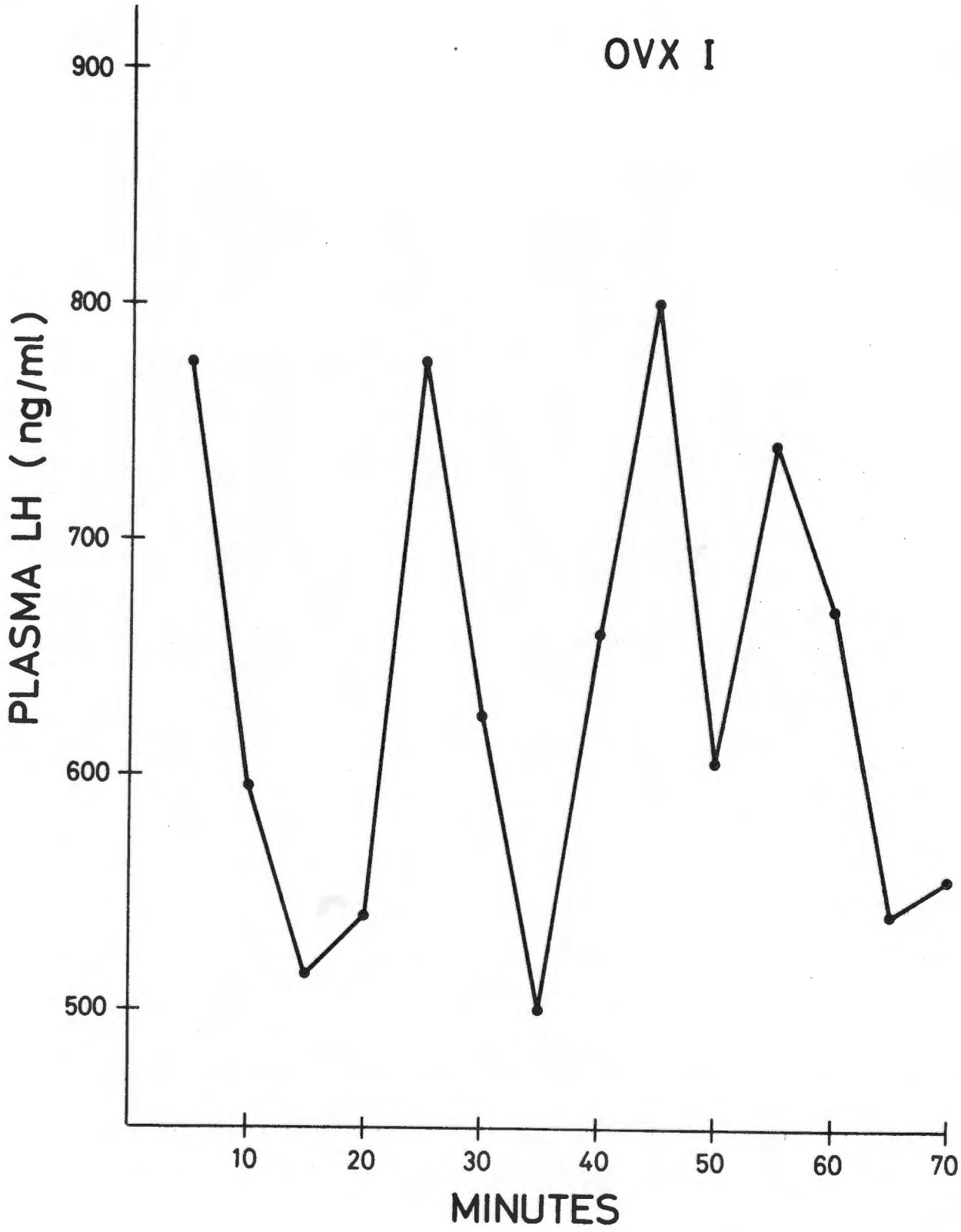


FIG 17 Pulsatile LH release in conscious, ovariectomized rat I

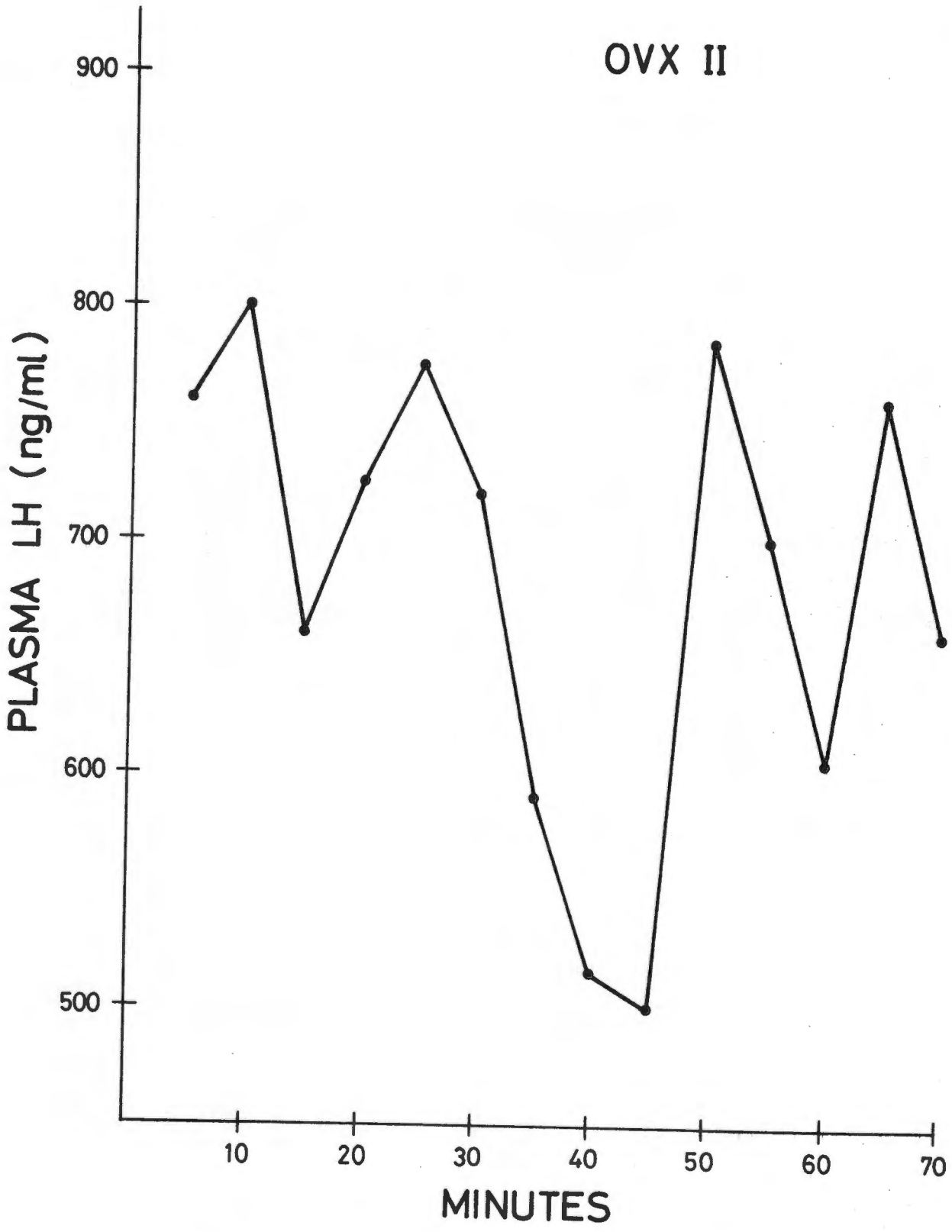


FIG 18 Pulsatile LH release in conscious, ovariectomized rat II

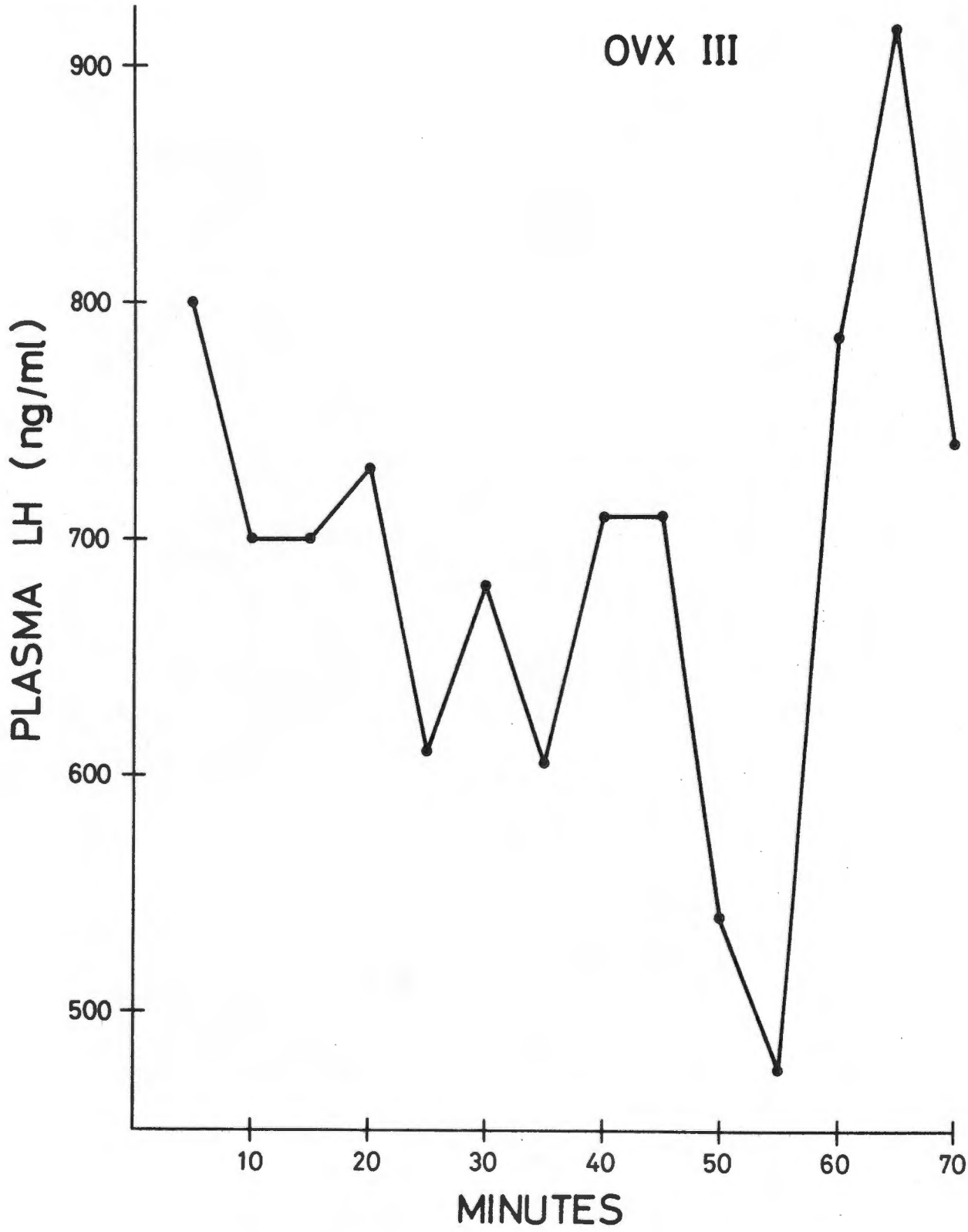


FIG 19 Pulsatile LH release in conscious, ovariectomized rat III

S E C T I O N 2

THE EFFECT OF AUDIOSTIMULATION ON LH SECRETION IN CONSCIOUS MALE AND IN NORMAL AND OVARIECTOMIZED FEMALE RATS

Males Twelve studies were performed using chronically cannulated male rats (300-350 grams). An attempt was made to correlate plasma LH concentration both during and after acoustic exposure with the various frequency/intensity combinations of the audiostimulus.

The total duration of the sound treatment (3 sessions) and the "on" (20secs) / "off" (40secs) sequence were maintained constant at all times. A "session" consisted of intermittent audiostimulation extending between 8pm and 6am, and again between 8am and 10am.

Each experiment lasted for 9 days and was conducted as follows: Blood was sampled continuously by peristaltic pump, from the first group of rats, for 1 hour on each of 4 consecutive days. (Pump flow rate was constant, but varied between collections from 1.0 to 1.5 ml/hour). Blood sampling began each day between 10.15 and 10.45am, and the audiostimulation program commenced on the evening of day 1. The above procedure was repeated with a second group of rats on days 5 to 8. No blood was sampled on day 9. Instead, the sound frequency and intensity were adjusted to satisfy the requirements of the next experiment, and the first group of rats introduced into the sound room at midday in readiness for the following experiment. (TABLE .9..) In addition, after each series of experiments at a particular sound intensity blood samples were obtained from all animals on each of 4 consecutive days in the absence of the audiostimulus. Each animal acted as its own control, against which to compare

TABLE ..9.. RANDOM DISTRIBUTION OF EXPERIMENT NUMBERS (UNDERLINED) BETWEEN THE VARIOUS FREQUENCY / INTENSITY COMBINATIONS TESTED

		Frequency (KHz)		
		10	5	2.5
Intensity (db)	80	4	8	7
	70	1	6	3
	60	5	9	10

Experiments 2, 11 and 12 acted as controls for the experimental procedure.

the effects of the treatment and the effects of the experimental procedures (without the audiostimulus) on the LH response. Haematocrit and haemoglobin concentration of blood from each rat was estimated on days 1 and 4 of experiments 2,4,6 and 8,10,12 respectively.

At the conclusion of the study, plasma samples from all experiments were included in a single assay.

Plasma LH concentrations (TABLE ..10..) are shown for each animal

a) during the sound treatment study; and
b) during the control studies.

Mean plasma LH concentrations and standard deviations have been calculated for each of the sound-treatment and control days and are shown in TABLE ..11.. These results are depicted graphically in FIGS ..20.. , ..21.. and ..22.. Statistical analysis of results, involving the use of the paired Student's t-test, revealed no significant differences between plasma LH concentrations

a) on any sound-treatment day as compared with the corresponding control sampling day; and
b) between day 1 and either days 2,3 or 4 in any given experiment.

Haematocrit values of blood on days 1 and 4 were not significantly different although there was a downward trend towards the fourth day, while haemoglobin concentrations were significantly lower on day 4 ($P < 0.001$). See TABLES ..11.. and 12..

TABLE ....10 PLASMA LH CONCENTRATIONS (ng/ml) IN INDIVIDUAL MALE RATS ON EACH DAY OF THE SOUND-TREATMENT AND CONTROL EXPERIMENTS

Sound Treatment		RAT NOS.						$\bar{x}$	SD
		1	2	3	9	5	6		
80 db 10 KHz	Day 1	168	36	64	82	28	26	67.3	54.0
	2	135	44	72	57	31	16	59.2	42.0
	3	102	25	62	66	19	6	46.7	36.2
	4	123	35	73	56	35	21	57.2	37.1
Nil	Day 1	69	35	58	50	25	25	40.1	17.1
	2	107	36	64	56	32	23	48.6	26.0
	3	69	38	44	49	31	28	41.0	13.3
	4	118	34	51	44	25	22	43.0	30.8
		1	2	8	9	5	10		
80 db 5 KHz	Day 1	117	37	20	54	20	62	51.7	36.3
	2	64	45	24	50	32	41	42.7	14.0
	3	169	34	24	60	29	45	60.2	54.8
	4	-	20	23	52	21	34	30.0	13.5
Nil	Day 1	69	35	24	50	25	37	36.2	15.0
	2	107	36	24	56	32	41	43.8	25.1
	3	69	38	22	49	31	24	36.4	15.2
	4	118	34	26	44	25	28	38.2	28.3
		1	2	8	9	5	10		
80 db 2.5 KHz	Day 1	76	40	30	51	30	63	48.3	18.6
	2	78	39	27	67	30	35	46.0	21.2
	3	70	34	22	35	31	56	41.3	18.0
	4	138	39	23	47	27	52	54.3	42.5
Nil	Day 1	69	35	24	50	25	37	36.2	15.0
	2	107	36	24	56	32	41	43.8	25.1
	3	69	38	22	49	31	24	36.4	15.2
	4	118	34	26	44	25	28	38.2	28.3

TABLE 10. (continued)

Sound Treatment		RATS.						$\bar{x}$	SD
		1	2	3	4	5	6		
70 db	Day 1	67	26	70	39	39	36	46.2	18.0
10 KHz	2	162	28	56	28	31	25	55.0	53.6
	3	108	28	55	49	33	20	48.8	31.8
	4	96	36	52	40	22	23	44.8	27.5
Nil	Day 1	69	35	58	48	25	25	38.6	17.6
	2	107	36	64	45	32	23	46.4	27.5
	3	69	38	44	24	31	28	37.0	14.3
	4	118	34	51	34	25	22	41.8	32.9
		1	2	8	9	5	10		
70 db	Day 1	107	37	24	44	30	45	47.8	30.1
5 KHz	2	113	35	29	58	28	58	53.5	32.2
	3	113	35	24	43	25	47	47.8	33.2
	4	92	48	29	52	28	28	46.2	24.9
Nil	Day 1	69	35	24	50	25	37	36.2	15.0
	2	107	36	24	56	32	41	43.8	25.1
	3	69	38	22	49	31	24	36.4	15.2
	4	118	34	26	44	25	28	38.2	28.3
		1	2	3	4	5	6		
70 db	Day 1	102	44	72	47	43	23	55.2	27.8
2.5 KHz	2	80	40	65	26	36	30	46.2	21.5
	3	32	41	43	22	37	17	32.0	10.5
	4	115	38	58	32	34	23	50.0	33.9
Nil	Day 1	69	35	58	48	25	25	38.6	17.6
	2	107	36	64	45	32	23	46.4	27.5
	3	69	38	44	24	31	28	37.0	14.3
	4	118	34	51	34	25	22	41.8	32.9

TABLE 10 (continued)

Sound Treatment		RATNOS.						$\bar{x}$	SD
		1	2	3	9	5	6		
60 db	Day 1	133	37	35	60	39	24	54.7	40.0
10 KHz	2	76	44	31	60	28	19	43.0	21.6
	3	70	32	26	57	39	24	41.3	18.4
	4	128	45	22	42	37	30	50.7	38.8
Nil	Day 1	69	35	58	50	25	25	40.1	17.1
	2	107	36	64	56	32	23	48.6	26.0
	3	69	38	44	49	31	28	41.0	13.3
	4	118	34	51	44	25	22	43.0	30.8
		7	2	8	9	5	10		
60 db	Day 1	25	30	30	42	36	20	31.6	8.2
5 KHz	2	30	44	37	48	30	30	37.8	8.1
	3	45	31	29	58	20	30	33.6	14.3
	4	34	38	25	50	34	43	38.0	9.4
Nil	Day 1	-	35	24	50	25	37	32.9	10.9
	2	-	36	24	56	32	41	36.8	12.4
	3	-	38	22	49	31	24	32.8	10.6
	4	-	34	26	44	25	28	30.2	10.5
		11	2	8	9	5	10		
60 db	Day 1	51	43	34	74	26	37	44.2	16.9
2.5 KHz	2	41	25	25	62	26	37	36.0	14.4
	3	17	29	30	36	24	32	28.0	6.1
	4	37	-	23	51	23	36	34.0	11.7
Nil	Day 1	30	35	24	50	25	37	32.3	10.5
	2	23	36	24	56	32	41	34.2	14.1
	3	17	38	22	49	31	24	29.9	11.6
	4	19	34	26	44	25	28	28.3	10.5

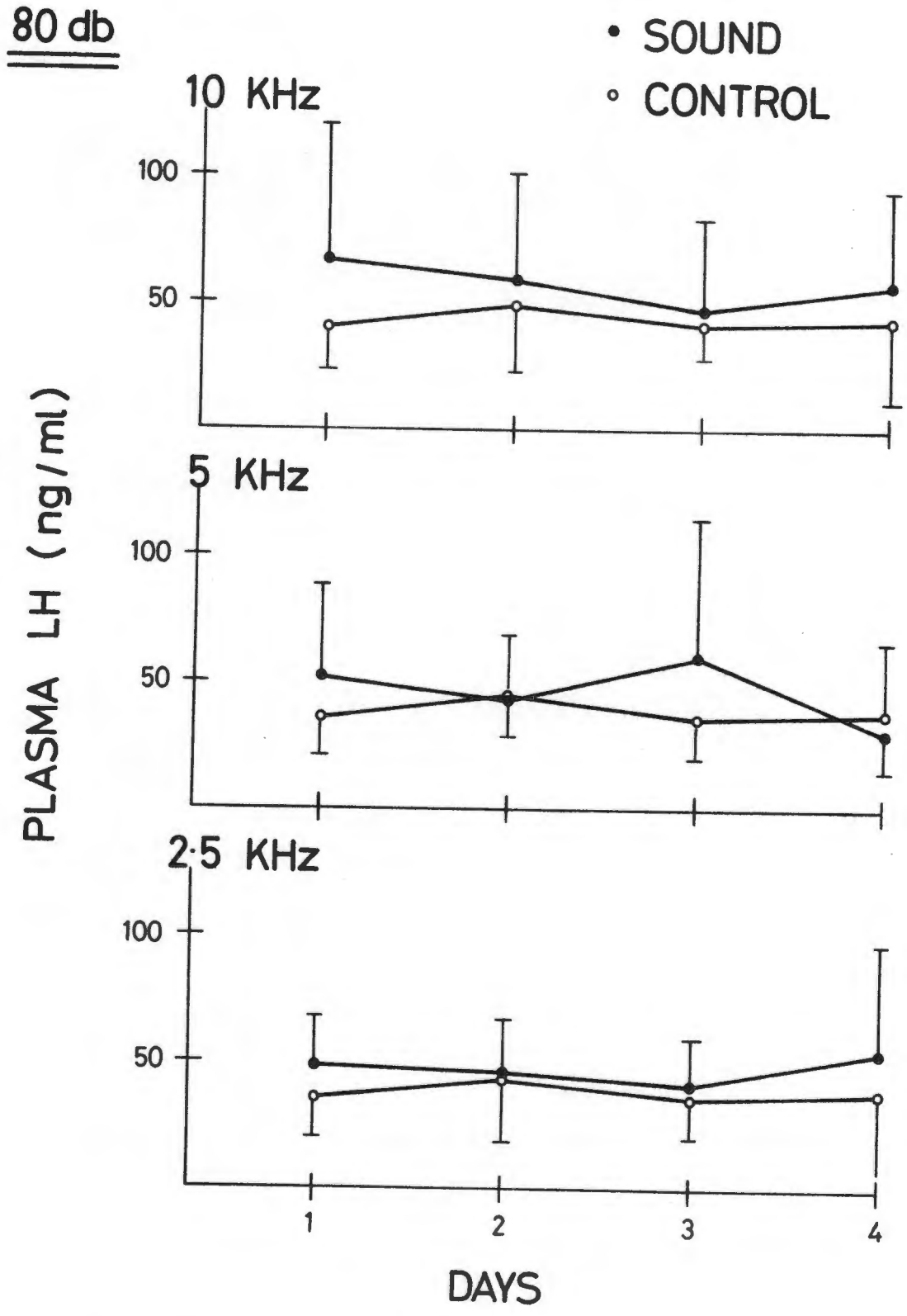


FIG 20 Mean plasma LH concentration (±SD) in conscious male rats exposed to 80db sound at 2.5, 5.0 and 10.0 KHz

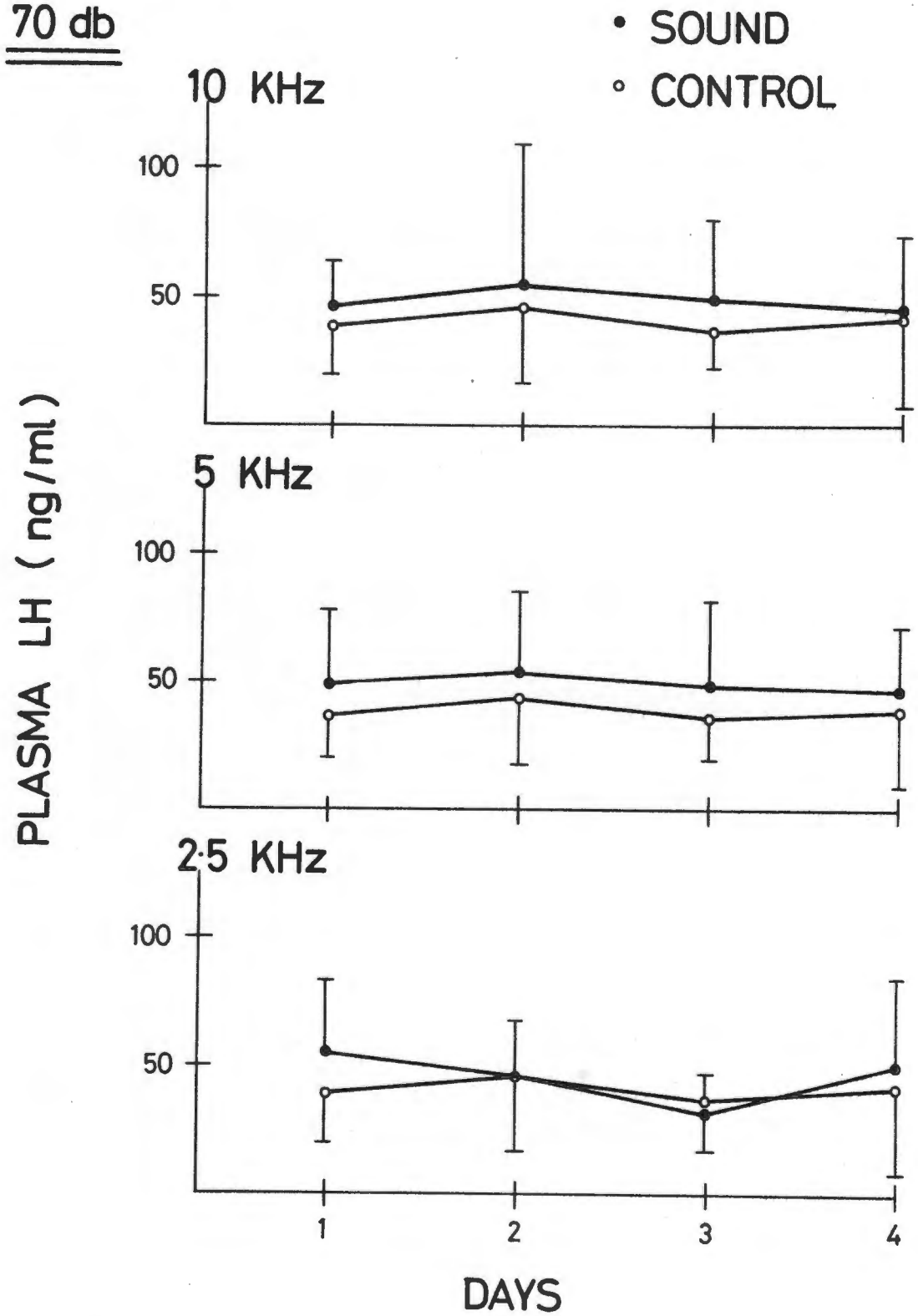


FIG 21 Mean plasma LH concentration (±SD) in conscious male rats exposed to 70db sound at 2.5, 5.0 and 10.0 KHz

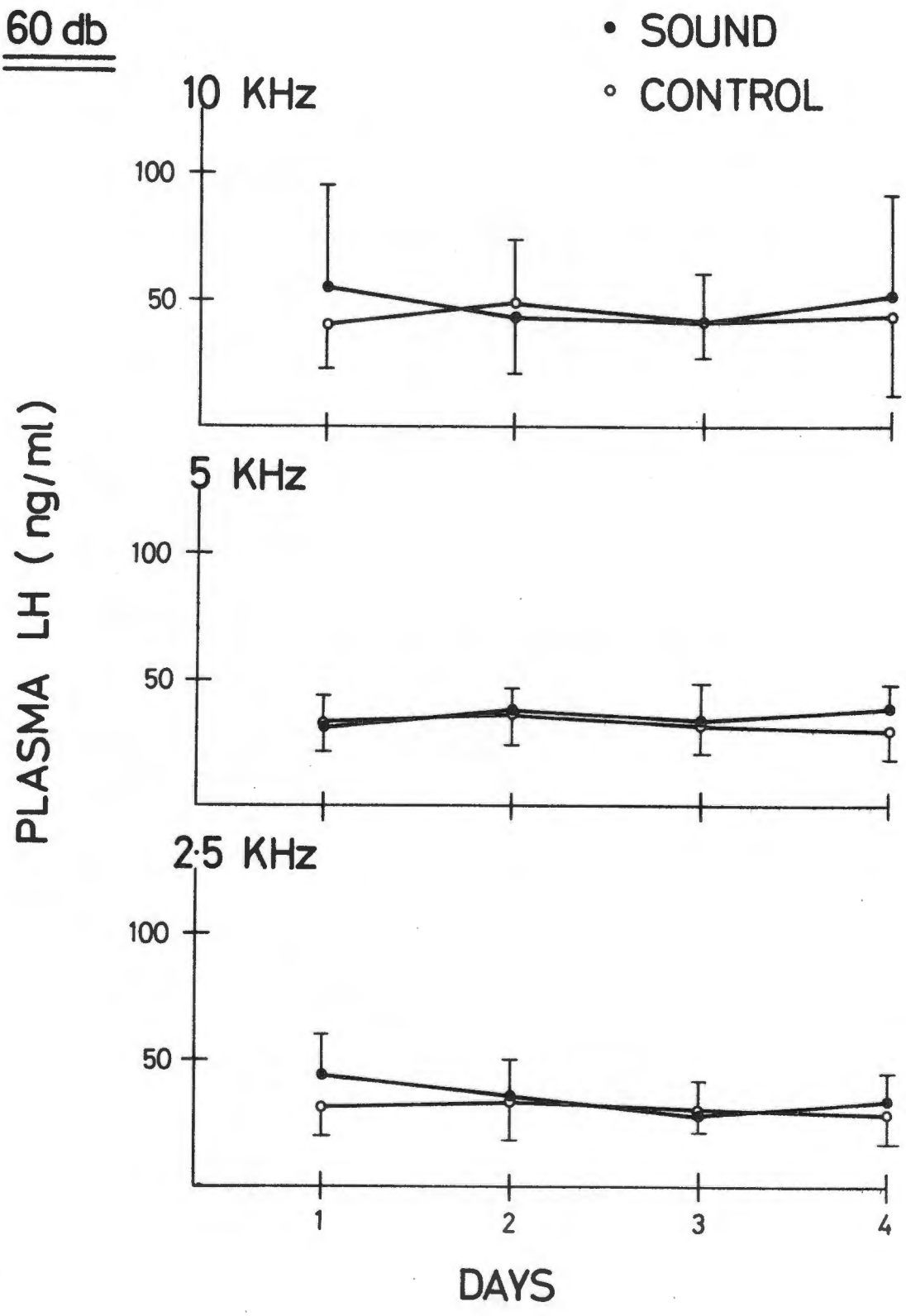


FIG 22 Mean plasma LH concentration (±SD) in conscious male rats exposed to 60db sound at 2.5, 5.0 and 10.0 KHz

TABLE ..11 HAEMATOCRITS OF BLOOD SAMPLES ON DAYS 1 & 4 OF THE MALE AUDIOSTIMULATION EXPERIMENTS

Experiment No.	Sound	Rat Nos.	HAEMATOCRIT (%) Day 1	Day 4
2	0 KHz/0db (control)	1	46	54 *
		2	45	43
		3	43	45
		4	49	45
		5	47	44
		6	48	44
4	10 KHz/80db	1	50	45
		2	44	44
		3	57 *	46
		9	41	45
		5	46	43
		6	42	42
6	5 KHz/70db	1	49	44
		2	44	42
		8	40	41
		9	43	42
		5	47	48
		10	48	47
		n	16	16
		$\bar{x}$	45.4	44
		SD	3.1	1.9
Paired t-test:		$\bar{d}$	1.38	
		Sd	2.6	
		t	2.1117	
		P <	0.1	

* Values outside the 95% confidence limits were rejected in accordance with the statistical method of Dixon (49).

TABLE ..12 HAEMOGLOBIN CONCENTRATIONS OF BLOOD SAMPLES ON DAYS 1 and 4 OF THE MALE AUDIOSTIMULATION EXPERIMENTS

Experiment No.	Sound	Rat Nos.	HAEMOGLOBIN CONC. (g%) Day 1	Day 4
8	5 KHz/80db	1	-	-
		2	13.7	12.5
		3	13.0	13.5
		9	13.0	12.9
		5	12.8	12.8
		6	13.5	12.5
10	2.5 KHz/60db	11	16.2	13.8
		2	-	-
		8	13.9	11.9
		9	14.5	12.5
		5	13.5	12.2
		6	13.8	11.2
12	0 KHz/0db (control)	11	14.0	12.6
		12	14.2	12.5
		8	14.1	12.3
		9	12.9	12.2
		5	14.5	12.5
		10	14.8	14.2
		n	16	16
		$\bar{x}$	13.9	12.6
		SD	0.9	0.7
	Paired "t" test:	$\bar{d}$	1.27	
		Sd	0.9	
		"t"	5.6518	
		P <	0.001	

Ovariectomized Females

Three adult female rats (300 grams) which had been bilaterally ovariectomized 4 months earlier, were anaesthetized and indwelling jugular cannulae placed as described on page 90. Four studies were carried out to investigate the possible effects of different sound frequencies (with constant intensity of 80db) on plasma LH concentration.

Experiment 1: frequency 10 KHz
2: 2.5 KHz
3: 5 KHz
4: 0 KHz (control study - no sound). designated OKHz in FIG 23

The experiments were conducted in the same manner as described for the male rats (page 110) except that blood was sampled only on days 1 and 4 of each experiment - i.e. before and after the 3 sessions of sound treatment. On/off sequence and total duration of exposure to the audiostimulus were maintained constant throughout all these studies. (see Male sound study, page 110)

Results are shown in FIG 23 (see Appendix Table 21.) In order to analyse these results, paired Student's t-tests were performed using the differences in plasma LH concentrations between

a) days 1 and 4 in each study;
b) day 1 in the sound-treated group and day 1 in the control group; and
c) day 4 in the sound-treated group and day 4 in the control group.

The only statistically significant effect noted in these studies was the elevation of plasma LH concentration on day 4, in the 5KHz/80db group, as compared with values

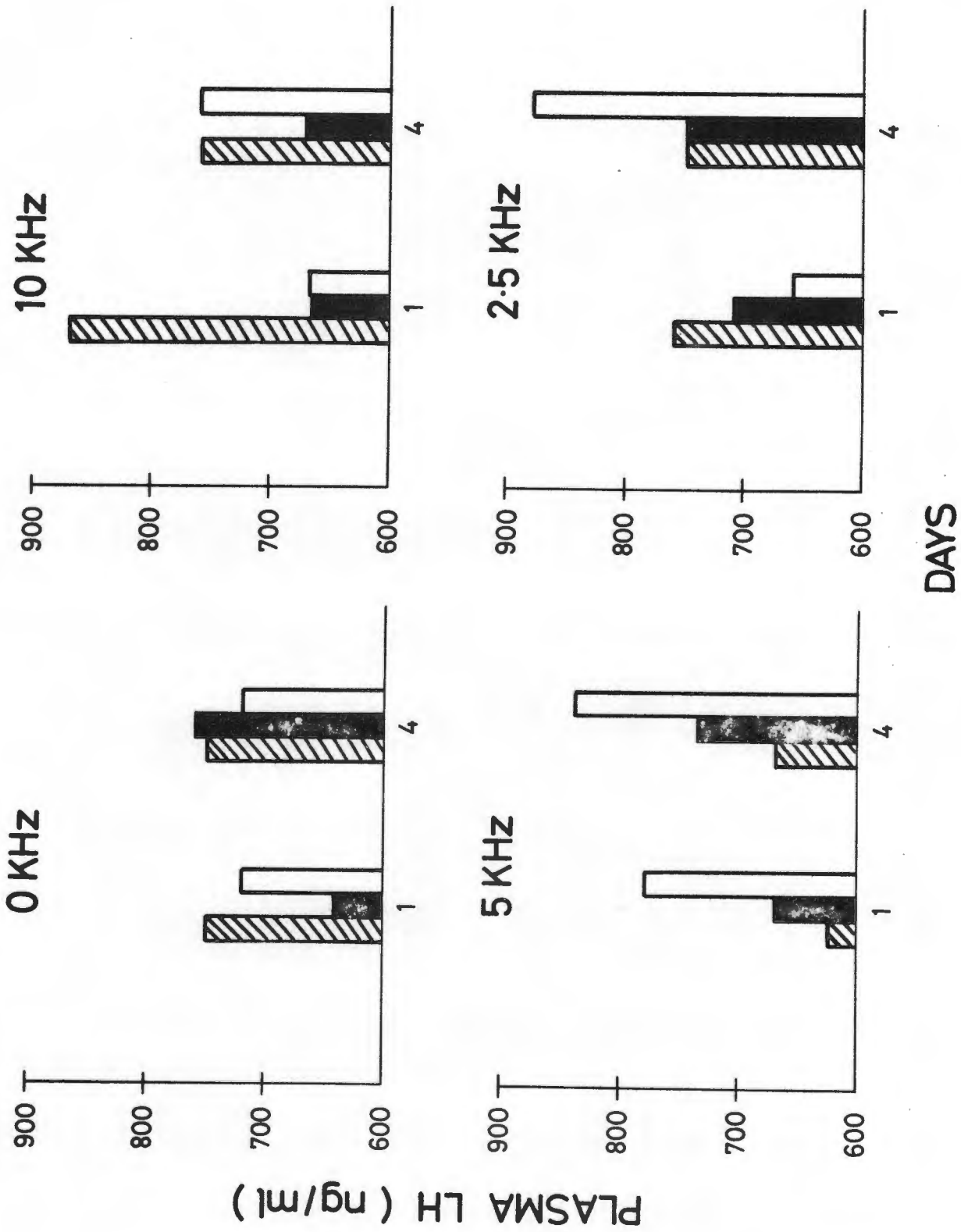


FIG 23 Mean plasma LH concentration in ovariectomized rats before and after exposure to an 80db acoustic stimulus

on day 1 before the sound treatment ($P < 0.02$). However, neither LH concentration on day 1 nor on day 4 of this treatment group differed significantly from LH levels on the corresponding days of the control study.

ADDENDUM:

Preliminary observations on the effects of audiostimulation on plasma LH concentrations were made in 2 intact female rats.

The chronically cannulated animals were exposed to a 10 KHz/65db audiostimulus with on/off sequence and total duration of sound exposure as described in the earlier studies. Blood was sampled by peristaltic pump for 1 hour on each of 4 consecutive days. Results (see FIG 24. and Appendix Table 22) suggest facilitation of LH secretion between days 2 and 4 of the audiostimulation program. Mean plasma LH concentration increased from 157.5 ng/ml on day 1 to 218.5 ng/ml on day 4.

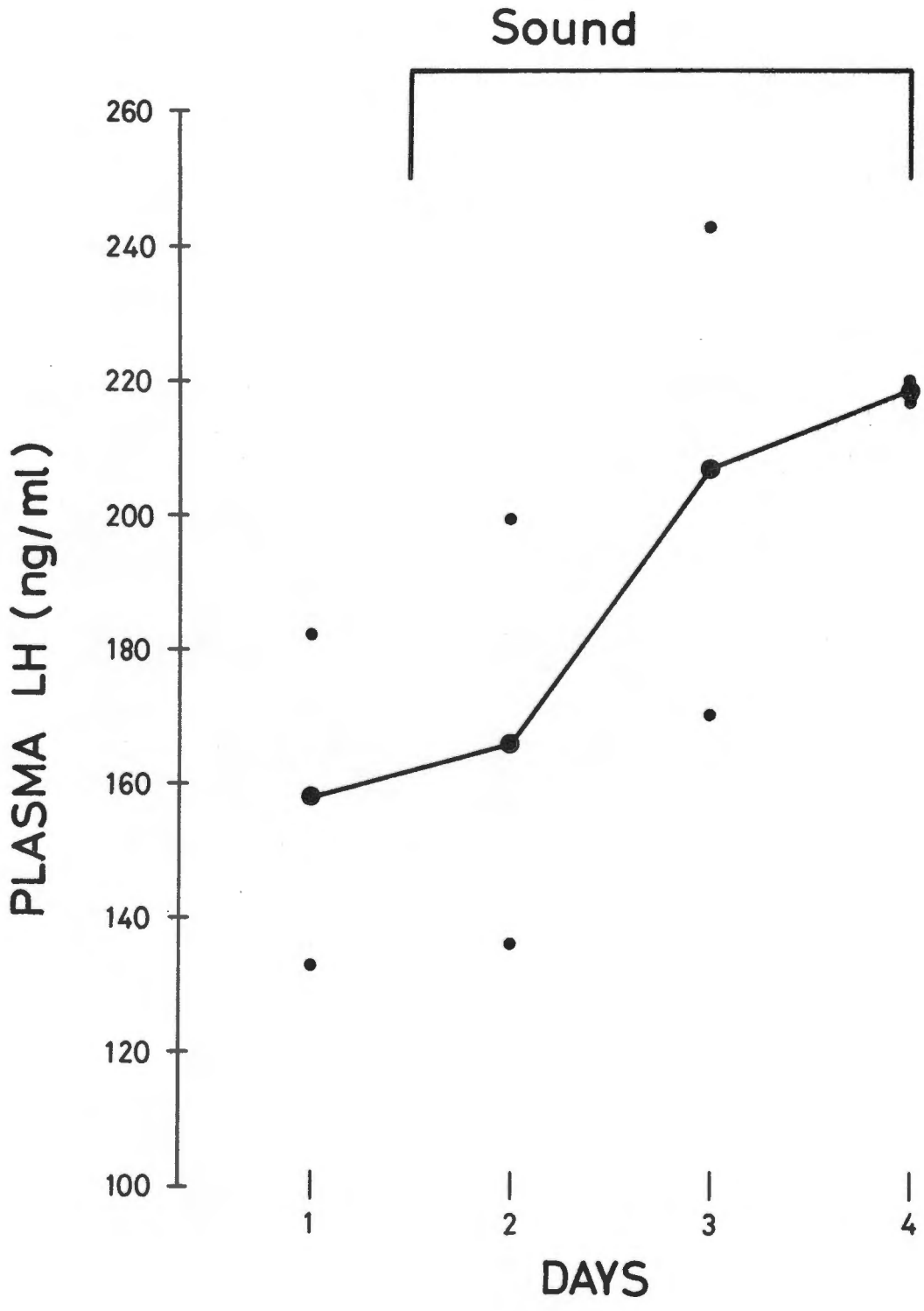


FIG 24 Mean plasma LH concentration in intact female rats on each day of the sound stimulation program

S E C T I O N 3

PLASMA LH CONCENTRATIONS IN CONSCIOUS MALE AND OVARIECTOMIZED FEMALE RATS FOLLOWING RAPID INTRAVENOUS INJECTION OF LRH

Males Five studies were carried out, in a random sequence, using 5 adult rats (300-320 grams) bearing indwelling jugular cannulae (see page 90.). The animals were heparinized (500 Units heparin) as described on page 95. Following the aspiration of the first blood sample (at time zero), 0.2ml saline containing the desired quantity of LRH was rapidly injected intravenously, and subsequent blood samples (0.4ml) were aspirated at 5, 10, 20, 40 and 80 minutes after LRH administration.

This procedure was followed using 5 different doses of LRH (doubling dilutions of LRH extending between 800 and 50ng LRH per 0.2ml saline). In a control study 0.2ml saline, containing no LRH, was injected into each of two animals. The animals had a rest period of approximately 4 days between each study.

Curves reflecting changes in mean plasma LH concentration, at specific times after administration of LRH (or saline), are depicted in FIG 25 (see Appendix Table 23.) The arrow at zero time indicates rapid intravenous injection of LRH (or saline) immediately after aspiration of the first blood sample.

Control plasma LH concentration, prior to LRH injection, for all experiments was 52.7 ± 14.2 ng/ml. At 50 and 100ng doses of LRH, peak plasma LH concentrations of 202.4 ± 44.1 ng/ml and 246 ± 105 ng/ml respectively, were attained 10 minutes after LRH administration. These peak values were significantly

elevated above those at zero time ($P < 0.001$ and 0.02 respectively. Thereafter plasma LH level declined exponentially to approach the resting level after 80 minutes. (From the shape of the apical region of the 100ng LRH curve, it appears that the peak plasma LH concentration might have occurred between 10 and 20 minutes after LRH injection). Higher doses of LRH (200, 400, and 800 ng) evoked peak plasma LH concentrations of 284 ± 99, 355.6 ± 164.6 and 393.2 ± 146.8 ng/ml respectively, 20 minutes after LRH administration. (All values were significantly elevated above the pre-injection values - $P < 0.01$, 0.02 and 0.01 respectively.)

In contrast to the LH-secretory response to the 50 and 100ng doses of LRH, plasma LH concentration still decreased exponentially but now remained somewhat elevated above the resting level after 80 minutes. (In all cases, plasma LH concentrations after 40 minutes were significantly greater than at time zero - see Appendix Table 23..)

Control studies, performed in an identical manner, revealed mean plasma LH concentrations either within or below the pre-injection range of the LRH-injected animals. The underlined circle at time 40 minutes (FIG 25.) indicates a value of less than 30ng LH/ml.

The dose-response curve shown in FIG 26. was derived by plotting peak plasma LH concentrations (Appendix Table 23.) against the dose of LRH injected. The curve has been presented in the conventional manner, with the dose of LRH plotted on a logarithmic scale. In this study the log dose-response relationship is linear in the dose range 50 to 800ng LRH per rat.

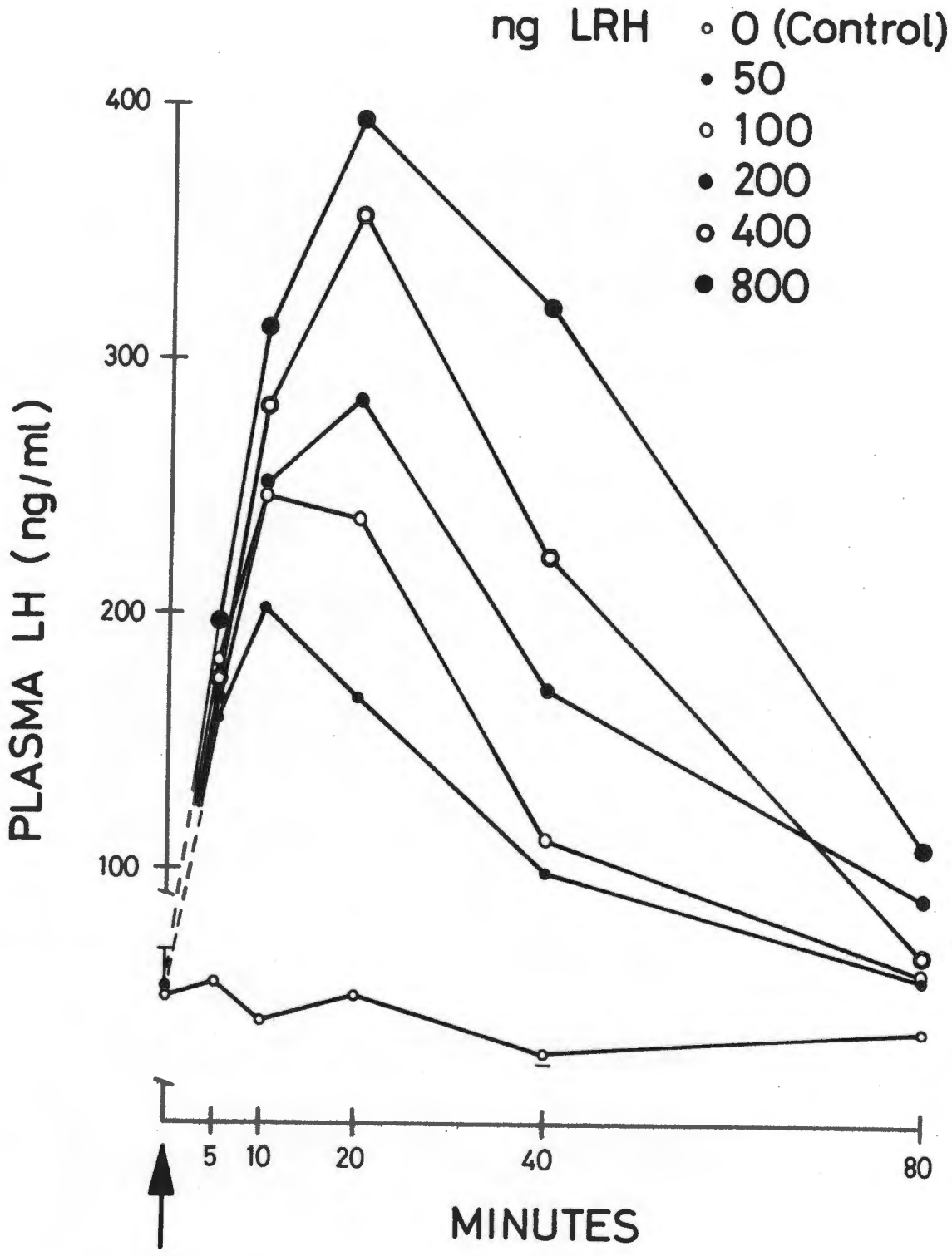


FIG 25 Mean plasma LH concentration in conscious male rats following intravenous injections of LRH, or saline.

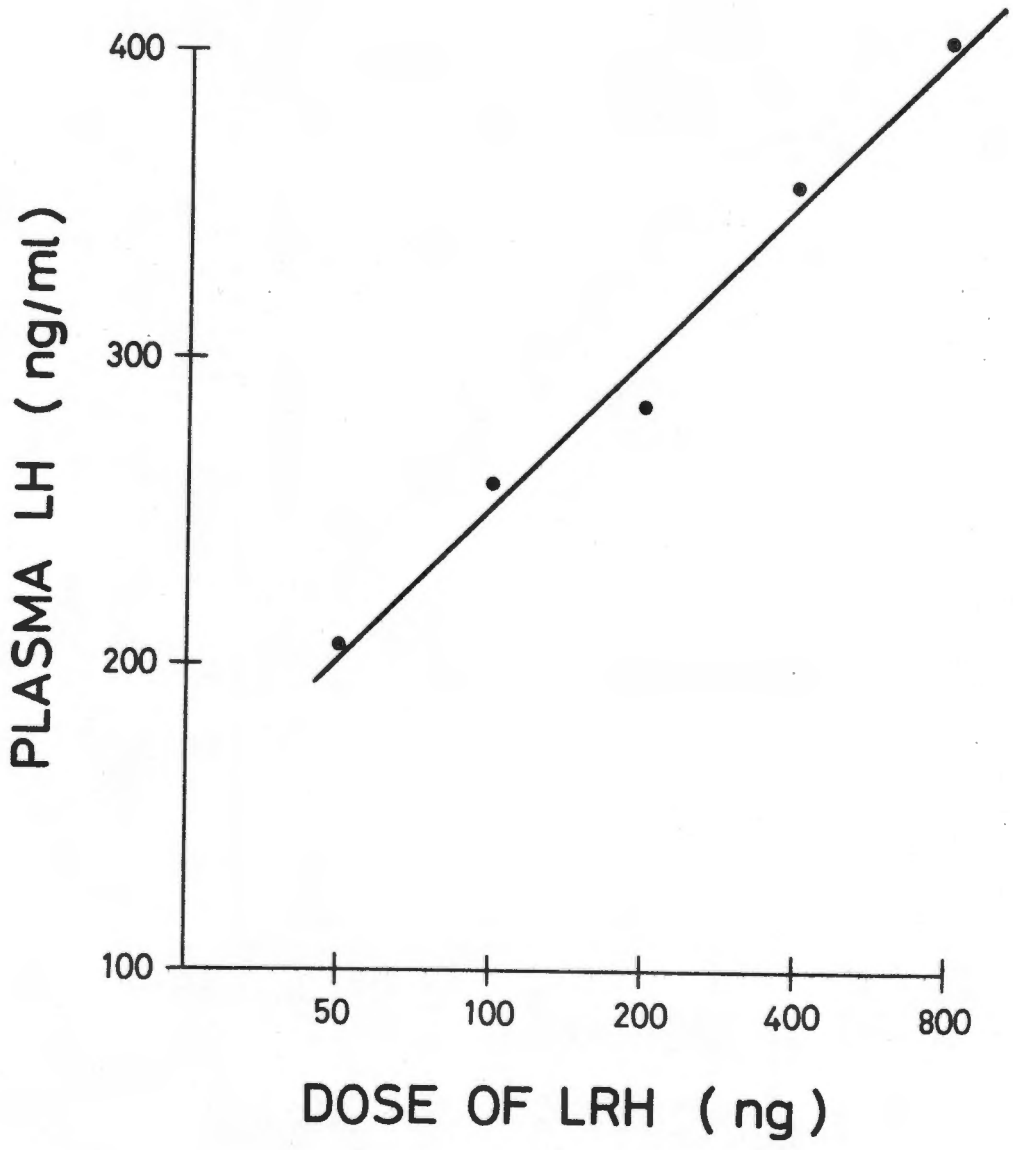


FIG 26 Log dose response relationship for LRH

Ovariectomized Females

Following the observed lack of effect of audiostimulation on plasma LH concentrations in chronically ovariectomized rats (page 121.), it was suggested that the pituitaries of these animals might be secreting LH at a maximal rate, thus preventing a possible audiostimulatory effect from manifesting itself. The aim of this study was to ascertain the extent of pituitary LH reserve in the same animals used in the earlier acoustic studies.

Following a 60 minute blood sampling period 800ng of LRH (in 0.2ml saline) was rapidly injected into each of the 3 rats and blood was again sampled continuously, by peristaltic pump, for an hour.

Plasma LH concentrations in these animals during each of the two 60 minute blood sampling periods are shown in FIG .27. (see Appendix Table .24.).
Mean plasma LH concentration, prior to LRH injection, was 743.3 ± 20.8 ng/ml. This level increased approximately 3-fold to 2,266.7 ± 86.2 ng/ml following LRH administration ($P < 0.001$).

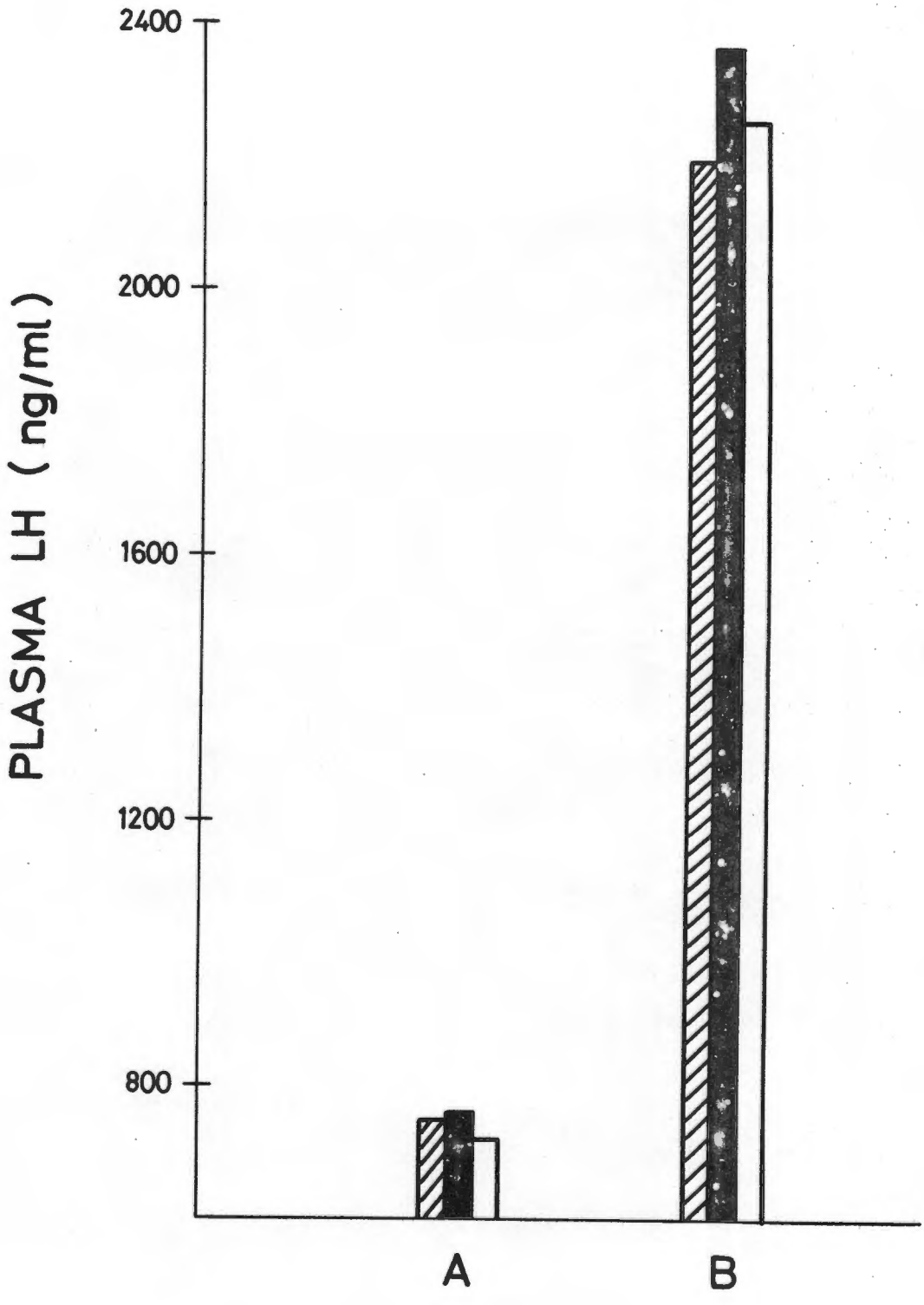


FIG 27 Mean plasma LH concentration in conscious, ovariectomized rats before (A) and after (B) intravenous injection of 800ng LRH

S E C T I O N 4

THE EFFECT OF ANAESTHETICS ON LH RELEASE

An investigation was conducted into the effects of anaesthesia on LH release with the aims of

1) comparing the effects of urethane and ether, and the barbiturates Sagatal (pentobarbitone sodium) and Inactin on serum LH concentrations in acutely cannulated male rats;

2) studying the effects of urethane anaesthesia on basal LH secretion in
   (a) acutely cannulated male rats;
   (b) chronically cannulated male rats;
   (c) acutely cannulated ovariectomized female rats;

3) studying the effects of urethane anaesthesia on the LH-secretory response to exogenous LRH in chronically cannulated male rats.

-----oOo-----

1) COMPARISON OF THE EFFECTS OF URETHANE, INACTIN SAGATAL AND ETHER ON SERUM LH CONCENTRATIONS IN ACUTELY CANNULATED MALE RATS

Twenty adult male rats (240-400 grams) were divided into 4 equal groups: A, B, C and D. Group A consisted of 10 control rats which were sacrificed by cervical dislocation, followed by collection of a single blood sample from each. Groups B, C and D each represented a group of test animals for one of the 3 anaesthetics selected - namely urethane, Inactin and Sagatal (see page 87 for doses).

All animals in a particular group were anaesthetized with the same anaesthetic and sequential blood samples drawn

from the left common carotid artery at 10-minute intervals (see page .95.).
The animals were sacrificed after 90 minutes by intra-arterial injection of an overdose of anaesthetic.

In addition, 4 male rats were subjected to 15 minutes of ether anaesthesia, following trilene induction, and sacrificed by severance of the right common carotid artery and right external jugular vein. Single blood samples were collected from these animals, and all sera were stored at -18°C while awaiting LH radioimmunoassay.

Serum LH concentrations at 10-minute intervals, extending between 30 and 90 minutes after intraperitoneal injection of the anaesthetic, are shown in FIG .28 (see Appendix Table.25) The curves illustrate an initial decline in serum LH concentration, with relatively constant LH levels being reached approximately 60 minutes after administration of the anaesthetic. This effect occurred with urethane, Inactin and Sagatal. However, the initial serum LH levels were markedly elevated in the urethane group and somewhat lower in the Sagatal and Inactin groups compared with control.

The mean serum LH values and standard deviations shown in TABLE .13. were derived using all points along the curves shown in FIG .28.
The table also includes mean serum LH concentrations in rats exposed to 15 minutes of ether anaesthesia, and in 9 rats sacrificed for control purposes (designated "Nil").
The data in TABLE ..13 demonstrate a significant facilitatory effect of urethane anaesthesia on serum LH concentration ($P < 0.025$), with about 41% elevation of serum LH level after

60 minutes, as compared with control animals. Conversely, Sagatal and ether anaesthesia depressed serum LH concentration ($P < 0.05$) by about 41% after 60 minutes and 49% respectively.

No statistically significant effect of Inactin anaesthesia on serum LH concentration was demonstrated.

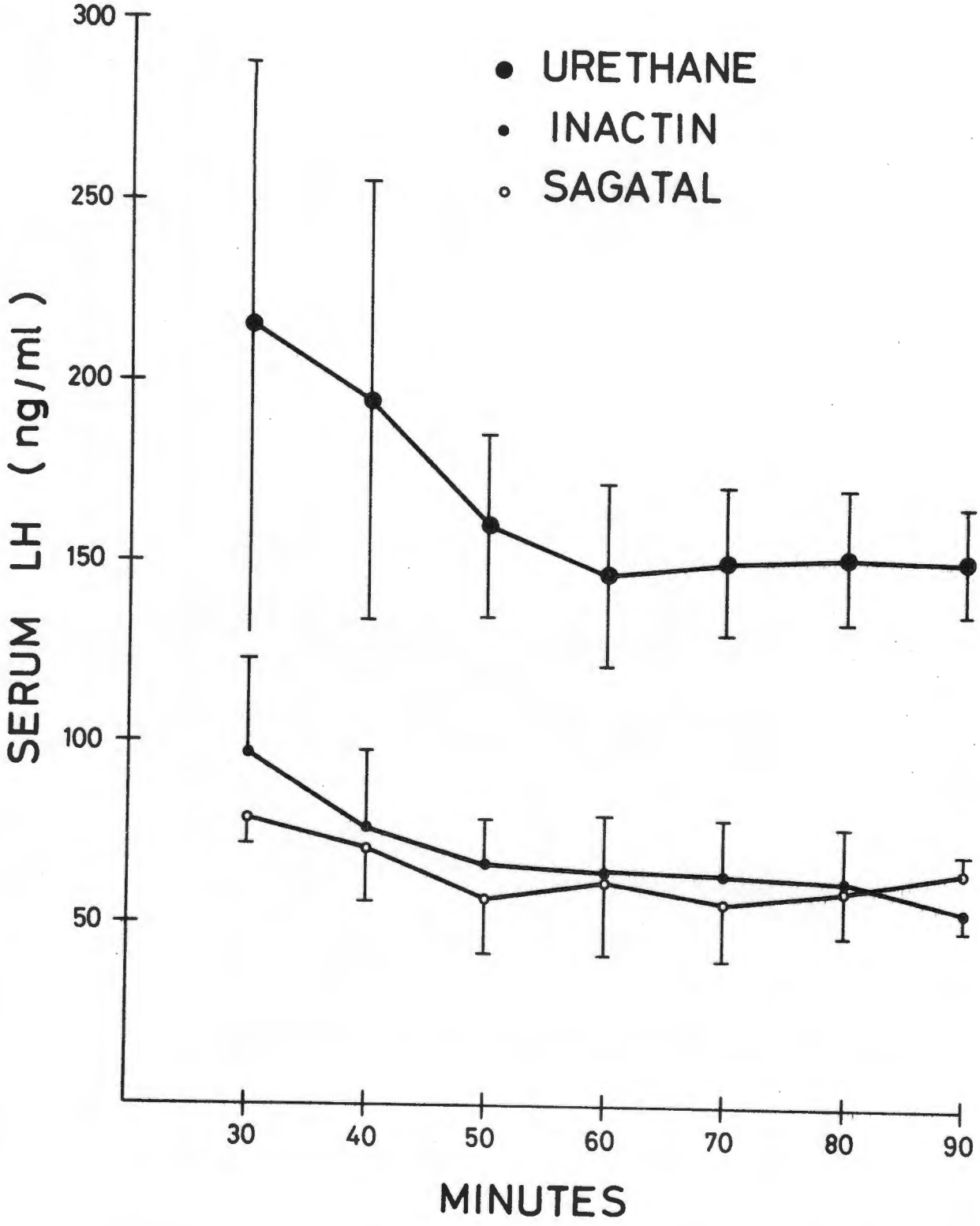


FIG 28 Mean serum LH concentration (±SD) in acutely cannulated male rats anaesthetized with urethane, Sagatal or Inactin

TABLE 13.

EFFECT OF THE ANAESTHETICS, URETHANE, INACTIN, SAGATAL AND ETHER ON RAT SERUM LH CONCENTRATION

Anaesthetic		Mean Serum LH (ng/ml)		SD
Urethane	(n=5)	166.6	**	35.8
Inactin	(n=5)	69.2		15.5
Sagatal	(n=6)	63.7	*	13.1
Ether	(n=4)	53.3	*	14.2
Nil	(n=9)	104.2		36.0

** = $P < 0.025$

* = $P < 0.05$

Compared with control animals sacrificed by cervical dislocation.

2) EFFECTS OF URETHANE ANAESTHESIA ON BASAL LH SECRETION

In order to investigate the effect of urethane on LH secretion, a study was performed using 9 adult male rats (300-350 grams) bearing indwelling jugular cannulae. The animals were heparinized with 500 Units heparin. Following the aspiration of 0.4ml blood (time zero), each rat was injected intraperitoneally with urethane and subsequent blood samples drawn 10, 20, 30 and 60 minutes thereafter. Thus, each rat acted as its own control. Plasma samples were stored at -18°C prior to LH assay (TABLE 14..).
Mean plasma LH concentration prior to urethane injection was 50.7 ± 23.7 ng/ml Ten, 20 and 30 minutes thereafter, mean plasma LH levels increased progressively to 69.6 ± 34.4, 73.0 ± 35.6 and 76.0 ± 33.3 ng/ml respectively. The values were all significantly greater than those at zero time (P values of <0.05, 0.02 and 0.01 respectively) using paired t-test. Sixty minutes after urethane injection, mean plasma LH concentration had decreased to a value not significantly different from the pre-injection level. However, since cervical dislocation (page 99..) and surgical stress (page 138..) appear to be associated with elevated serum LH levels, studies were performed both in the presence of and in the absence of surgical trauma, using groups of acutely cannulated and chronically cannulated animals respectively.

Acutely cannulated male rats

Four adult male rats (300-400 grams) were anaesthetized with urethane (see page .87.) and the right common carotid artery cannulated as described previously (page .89.).

TABLE 14

PLASMA LH CONCENTRATIONS IN CHRONICALLY CANNULATED MALE RATS AT SPECIFIC TIME INTERVALS AFTER ADMINISTRATION OF URETHANE

	Minutes after urethane injection				
	0	10	20	30	60
Rat No. 1	37	59	71	63	79 ng/ml
2	70	83	113	127	102
3	27	62	77	75	85
4	22	33	25	33	24
5	64	130	112	100	77
6	57	68	54	92	57
7	55	56	65	66	42
8	30	23	24	25	26
9	94	112	116	103	82
Mean LH	50.7	69.6	73.0	76.0	63.8
SD	23.7	34.4	35.6	33.3	27.8
		a	b	c	

a, b and c represent P values of less than 0.05, 0.02 and 0.01 respectively, compared with zero minute concentrations using paired Student's t-test.

Thereafter, 0.7ml blood samples were drawn at 30 and 60 minutes after administration of the anaesthetic, and the sera assayed for LH (TABLE .15.)

Mean serum LH concentrations, 30 and 60 minutes after urethane injection, were of the order of 180.3 ± 68.4 ng/ml. The relatively high concentration of LH in these animals may be associated with the anaesthetic effect or may be the consequence of surgical trauma due to the cannulation procedure. In the following study, the "stress" factor was eliminated and the effect on LH concentration of the anaesthetic alone was measured.

Chronically cannulated male rats

Six adult male rats (320-430 grams), bearing indwelling jugular cannulae, were anaesthetized with urethane and heparinized with 500 Units of heparin (see page .95.). Blood samples (0.4ml) were drawn 30 and 60 minutes after administration of the anaesthetic. Plasma samples were stored at -18°C prior to LH assay (TABLE .15.) The mean plasma LH concentration, 30 to 60 minutes after urethane injection was 76.2 ± 29.1 ng/ml. This concentration thus reflects an index of LH secretion, in the anaesthetized animal, in the absence of surgical trauma.

Serum LH concentrations in the acutely cannulated animals (180.3 ± 68.4 ng/ml) were highly significantly elevated ($P < 0.001$) above those in the chronically cannulated rats (TABLE .15.)

TABLE 15

EFFECT OF URETHANE ANAESTHESIA ON CIRCULATING LH CONCENTRATIONS IN BOTH CHRONICALLY AND ACUTELY CANNULATED MALE RATS

Chronically Cannulated		Acutely Cannulated
63 ng/ml		167 ng/ml
127		338
75		198
33		153
100		130
92		169
79		118
102		169
85		
24		
77		
57		
12	(n)	8
76.2	($\bar{x}$)	180.3
29.1	(SD)	68.4

"t" value 4.4356

$P < 0.001$

Acutely cannulated ovariectomized female rats

Two adult female rats weighing about 300 grams, which had been bilaterally ovariectomized 5 months earlier, were anaesthetized with urethane (see page ....87....) and the right external jugular vein of each cannulated. Following an equilibration period of 60 minutes, the animals were heparinized (500 Units of heparin) and blood sampled continuously by peristaltic pump from both rats for 75 minutes. Pump flow rate was 3.6 ml/hour. Five-minute fractions of blood (0.3ml) were collected during the sampling session and the plasma samples assayed for LH.

Results of this study are shown in FIG ....29.... (see Appendix Table ....26....)

Plasma LH concentrations fluctuated between 575 and 1345 ng/ml, with mean values in rats X and Y of 948 ± 115.6 ng/ml and 916 ± 251.8 ng/ml respectively. Statistical analysis of means and standard deviations revealed no significant difference between plasma LH concentrations in these animals (Appendix Table ....26....)

The release of LH in both animals followed an erratic pattern. Nevertheless a characteristic pulse of LH output was shown in one animal, where the hormone concentration rose from 720 to 1345 ng/ml in 15 minutes and declined to its minimum concentration of 575 ng/ml 50 minutes later. During the next 10 minutes the LH level rose again to 990 ng/ml. A similar but less dramatic rise and fall in LH concentration occurred in the second animal.

When compared with pulsatile LH release in the conscious ovariectomized animals, the duration of the pulses in the present study was clearly much longer (70 and 55 minutes

compared with 16.7 minutes in the conscious animals). Furthermore, statistical comparison of mean plasma LH concentrations in both groups revealed a significant elevation of the LH concentration ($P < 0.001$) in the anaesthetized group (see Appendix Table 27.)

Urethane anaesthesia, in the ovariectomized rats, thus appears to have induced a state characterized by a significant elevation of mean plasma LH concentration, and a considerable decrease in the frequency of the LH pulses as compared with values in the conscious, free-running animals.

However, it should be noted that the fluctuations in plasma LH concentration in the present study probably reflect the effects of both surgical trauma and urethane anaesthesia on the LH secretory mechanism.

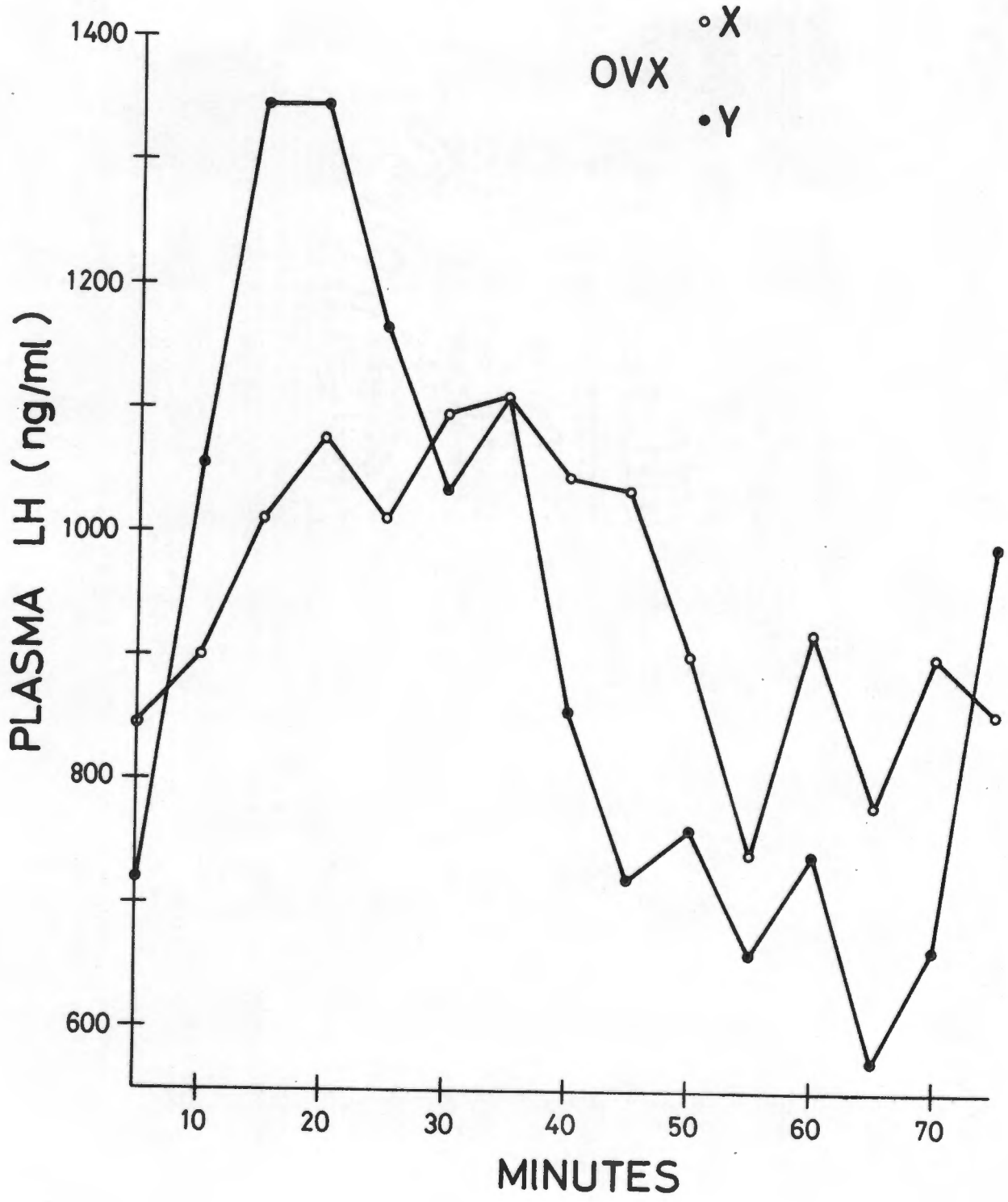


FIG 29 Variation with time in plasma LH concentration in acutely cannulated, ovariectomized rats (X and Y) anaesthetized with urethane

3) THE EFFECT OF URETHANE ANAESTHESIA ON THE LH-SECRETORY RESPONSE TO EXOGENOUS LRH IN CHRONICALLY CANNULATED MALE RATS

Having differentiated between the effects on plasma LH concentration of urethane anaesthesia, and of urethane anaesthesia plus surgical stress (previous pages), the present study was performed in order to define more precisely the influence of urethane per se on the hypothalamo-adenohypophysial mechanisms controlling LH secretion.

Eleven adult animals (320 -430 grams), bearing indwelling jugular cannulae, were used in the study. Rats 1 to 6 were anaesthetized with urethane and injected intravenously with 50ng LRH 60 minutes later. Three control rats (A, B & C) received 0.2ml saline in place of the LRH solution, while two more control rats received saline in place of the urethane injection.

One hour prior to urethane (saline) anaesthesia the animals were heparinized with 500 Units heparin. Immediately after aspiration of the first blood sample (0.4ml), 50ng LRH (or 0.2ml saline) was rapidly injected intravenously and subsequent blood samples drawn at the following times:

Experimental Rats

Conscious (I to V): 10, 40 and 80 minutes (after LRH or saline injection)

Urethane anaesthetized (1 to 3): 10 and 40 minutes
(4 to 6): 10, 40, 80 and 120 minutes

Control Rats

A, B & C : 10, 40, 80 and 120 minutes

1 and 2 : 10 and 40 minutes

Since blood samples were drawn from each animal before

and after LRH (or saline) injection, each rat acted as its own control.

Curves depicting mean plasma LH concentrations in urethane-anaesthetized rats, before and after intravenous injection of either 50ng LRH or 0.2ml saline, and in conscious animals following injection of 50ng LRH, are shown in FIG ..30. (see Appendix Table ..28.). The arrow at time zero indicates rapid intravenous injection of LRH or saline immediately after aspiration of the zero minute blood sample. In urethane-anaesthetized rats, injection of saline in place of LRH exerted no effect on plasma LH concentrations, while injection of 50ng LRH evoked a dramatic response:
Within 10 minutes of LRH injection, mean plasma LH concentration had increased from 70.7 ± 27.1 ng/ml at time zero to 281.2 ± 123.8 ng/ml ($P < 0.01$). This value was significantly greater than that in the saline-injected control group ($P < 0.05$).

In the conscious animals mean plasma LH concentration reached a peak value 10 minutes after LRH injection, while peak concentrations in the urethane-anaesthetized group (350 ± 128 ng/ml) were attained 40 minutes after LRH administration. This peak concentration was significantly higher than the 40-minute plasma LH concentrations in both the control (saline in place of LRH) group ($P < 0.02$) and in the conscious LRH-treated animals ($P < 0.005$). Mean plasma LH level then declined exponentially to 81.5 ng/ml at 120 minutes, which was similar to that in the conscious animals at 40 minutes see TABLE ..16.)
Plasma LH concentrations, after LRH stimulation, in the urethane-anaesthetized animals differed from those in conscious rats in several ways:

a) Higher LH levels were attained, although the time after LRH injection at which the peak occurred was considerably later in the anaesthetized rats.

b) The exaggerated LH-secretory response to 50ng LRH in urethane-treated rats was equivalent to the administration of about 400 ng LRH to conscious rats.

c) The basal level of LH was reached only 120 minutes after LRH administration compared to 80 minutes in conscious rats.

Furthermore, the possible effects on plasma LH level of intraperitoneal injection of fluid was tested in a second control study. Two rats were injected intraperitoneally with volumes of saline equivalent to the mean volumes of urethane solution used (see Appendix Table 28.). Mean plasma LH concentrations prior to, and after, injection of 50 ng LRH did not differ significantly from mean concentrations in the conscious animals.

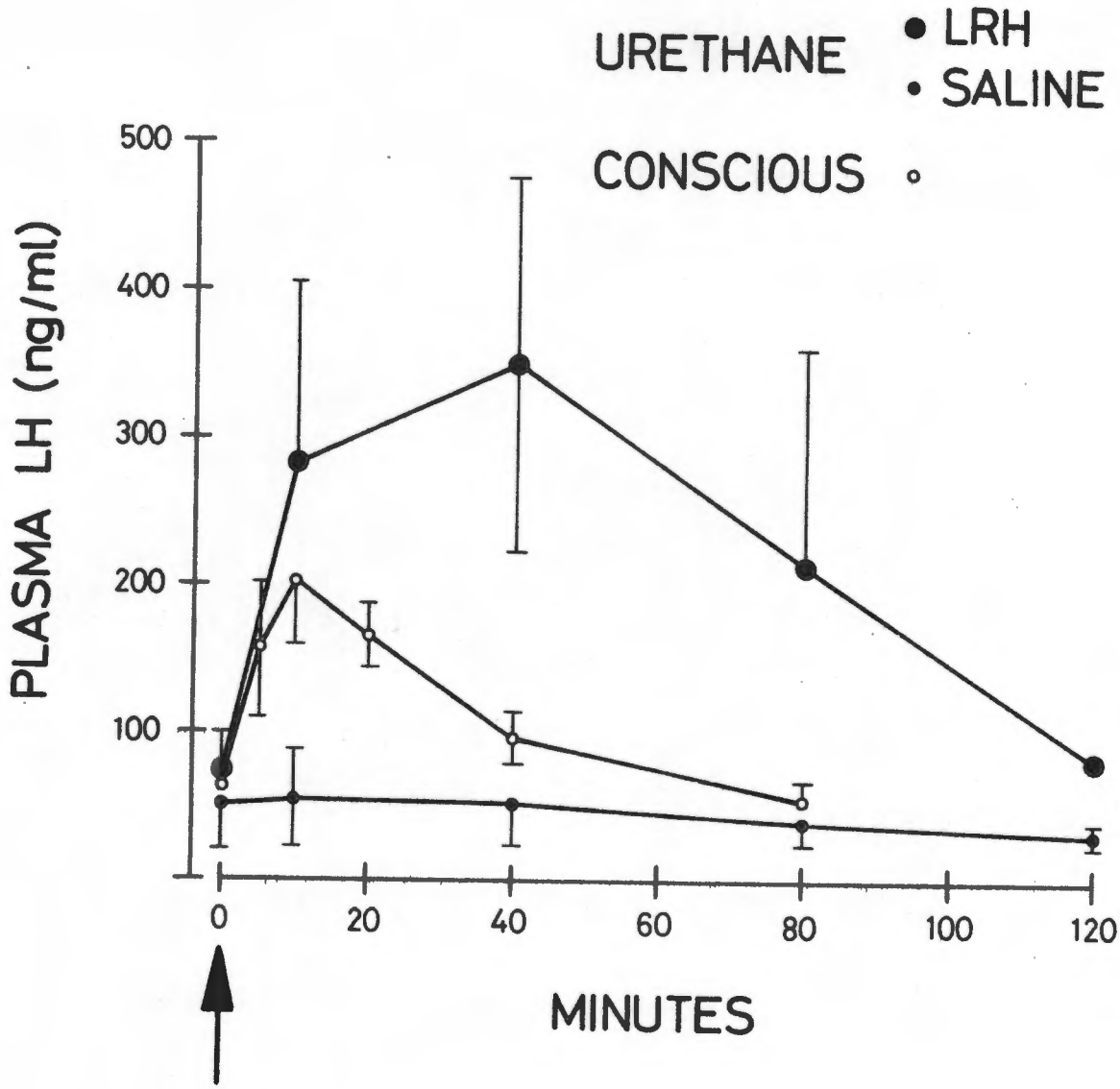


FIG 30 Mean plasma LH concentration (±SD) in urethane-anaesthetized and conscious male rats after intravenous injection of 50ng LRH, and in saline-injected control animals

TABLE 16

MEAN PLASMA LH CONCENTRATIONS (ng/ml) IN CONSCIOUS AND IN URETHANE-ANAESTHETIZED MALE RATS FOLLOWING INTRAVENOUS LRH ADMINISTRATION

Treatment		Minutes after LRH or saline				
Anaesthetic	Releasing Hormone	0	10	40	80	120
Urethane	LRH 50ng	70.7	281.2	350	212	81.5
Nil	LRH 50ng	62.8	202.4	98.2	57	
Nil (saline)	LRH 50ng	62.5	214.5	115.5		
Urethane	Nil (saline)	50	54.3	52.7	41.3	35

CHAPTER 5

DISCUSSION

Chapter 5

RAT LH RADIOIMMUNOASSAY

The radioimmunoassay technique used in these studies was found to be particularly useful for repetitive estimations of LH in individual rats. This "ultramicro" aspect of the assay enabled studies, involving sequential blood sampling from the same rat, to be carried out with minimal adverse effects secondary to hypovolaemia. In addition, the 0.4ml blood samples aspirated yielded sufficient plasma (or serum) for duplicate estimation of LH concentration. The sample volume could be reduced still further in studies with ovariectomized animals, since unknown sera were diluted 1 in 5 with buffer prior to LH assay. It was therefore possible to aspirate 0.1ml blood samples from such animals, and still have sufficient serum or plasma for the LH assay.

The sensitivity of the assay was found to be 16ng LH/ml. This value is in agreement with the work of Seki *et al* (195) who reported a sensitivity of 15ng LH/ml, using the same standard LH preparation as in the present study. It should be noted, however, that the sensitivity of the assay system, while being adequate for this study, could be further increased by using a greater dilution of first antibody.

Reproducibility of the assay system was quantified by determining the coefficients of both inter-assay variation (± 6.6%) and intra-assay variation (± 3.1%). Both indices of reproducibility were found to agree with the findings of Naftolin and Corker (152) and Seki *et al* (195) who reported

coefficients of inter-assay variation of ± 7.5% and ± 12.7% respectively. Furthermore, the coefficient of inter-assay variation using pooled serum specimens (± 6.4%) compared favourably with the value reported by Naftolin and Corker (152) in their original ultramicro assay system (± 7.5%).
In order to eliminate errors secondary to inter-assay variation, all unknown samples from a particular experiment were included in a single assay. Results of independent studies were compared only when the inter-assay variation of the internal standard serum sample was within the limits described above.

In addition, the demonstration of parallelism between unknown sera and standard LH has added further weight to the validity of the assay procedure, by indicating comparable immunological behaviour of the unknown and standard solutions.

The assay system was also characterized by measurement of serum LH concentrations following bilateral ovariectomy and, after the cannulation procedure had been established, during the normal oestrous cycle of the rat. The findings of these studies, which have been presented in Chapter 4 and will be discussed in the following pages, are in general agreement with the well-documented observations of other workers. Furthermore, plasma LH concentrations could be measured over a considerably wide range of values, extending from about 40ng LH/ml in conscious male rats, to about 2,500ng LH/ml in conscious ovariectomized animals injected with LRH.

On the basis of these findings, the present rat LH radioimmunoassay may be considered to compare favourably with other reported radioimmunoassays with respect to the reliability criteria, and to possess the added advantage of requiring

minimal volumes of unknown solution.

BASAL SECRETION OF LH

The results of those investigations aimed at establishing basal levels of LH secretion have clearly demonstrated the dependence of plasma LH concentration on the method of blood collection employed. While it is difficult to obtain a true reflection of circulating LH levels in intact, normal rats, the technique of chronic cannulation has permitted the aspiration of blood samples from conscious, unstressed animals several weeks after surgery.

It is reasonable to suggest that this method would provide more accurate estimation of basal plasma LH concentrations than the less sophisticated methods tested. Given that this is so, cervical dislocation clearly increases the basal concentration very dramatically. Since stress, following exposure of rats to ether vapour for three minutes, has been found to significantly elevate serum LH concentration (52) it is reasonable to suggest that the increased LH levels found after cervical dislocation could be induced by sudden trauma associated with the procedure. The increased sympathetic discharge known to occur at this time (22) may well contribute to the elevated LH levels.

This elevation of circulating LH concentration, secondary to cervical dislocation, was observed in both males and intact dioestrous females ($P < 0.001$ and 0.05 respectively) but failed to occur in the chronically ovariectomized animals. The latter observation could possibly be interpreted in the light of either (a) a small increment in serum LH level in the cervically-dislocated, ovariectomized rats, which could not be

detected in the assay, or (b) inability of the anterior pituitary gland to significantly increase LH output following cervical dislocation. These suggestions, however, must remain speculative until studies have been performed to define more precisely the factors influencing LH secretion in ovariectomized rats.

Plasma LH concentrations in chronically cannulated, unstressed animals (TABLE 8) have, by definition, been regarded as reflecting, most closely, the "normal" values, and are of the order of

Males	46.8 ±	27.2 ng/ml
Intact dioestrous females	77.9 ±	22.9 ng/ml
Chronically ovariectomized rats	701.7 ±	65.8 ng/ml

The studies with chronically cannulated, cycling female rats have clearly demonstrated the pro-oestrous rise in circulating LH levels, which has been well documented by other workers following both radioimmunoassay (147, 151, 158, 195) and bioassay (2, 175, 194) estimations of serum or plasma LH concentration. The present study has also demonstrated that serum LH concentrations at oestrus are significantly greater than at the dioestrous or metoestrous stages of the reproductive cycle ($P < 0.001$). While most workers have found serum LH levels to be low at all stages of the oestrous cycle other than the afternoon of pro-oestrus, some authors have reported serum LH levels to be somewhat elevated at oestrus (175, 194). The present observation is also in agreement with the results of previous work in these laboratories (117) and may therefore represent the normal pattern of LH secretion in the particular strain of rat used. The suggestion that this observation may be a consequence of a "stress effect" seems unlikely in view

of the fact that both dioestrous and metoestrous serum LH concentrations varied within narrow limits, and were in agreement with values reported earlier in this study (Appendix Table .15.). Furthermore, the highly reproducible nature of the rat LH radioimmunoassay system argues against the effect being an artifact introduced by the assay procedure.

Investigations made on ovariectomized rats have suggested that serum LH concentrations reach maximum levels 7 to 12 days post-operatively, while absolute levels reached were approximately 7-fold greater than those in the sham-operated dioestrous control animals ($P < 0.001$).
While the rise in serum LH concentration following castration is well established (166), considerable controversy still exists regarding the time course of this response. Seki et al (195) reported maximum serum LH levels being attained only 4 weeks after bilateral ovariectomy, with levels decreasing slightly after 8 weeks. The present study reflects only the acute response to ovariectomy (i.e. up to 3 weeks after operation). The results, however, do not appear to correspond with those of the latter investigators, since serum LH concentration would be expected to increase throughout the 4-week post-operative period, rather than to attain a relatively constant plateau after 12 days.
These findings are, however, in good agreement with those of Gay and Midgley (76) who have reported high serum LH levels 7 days after operation. Furthermore, the magnitude of the LH secretory response coincides with that found in the early study of McCann and Ramirez (136) who, using the ovarian ascorbic acid depletion assay of Parlow (165) as an index of LH activity, observed a 7-fold elevation of plasma LH concentration 2 to 3

weeks after castration. In addition, the authors suggested that the elevated plasma LH concentrations would persist for at least 12 months post-operatively - see (136).

More recently, Tapper et al (208) have demonstrated the dependence of the early response to ovariectomy in the rat on the reproductive state of the animal at the time of operation. The oestrous and metoestrous stages were characterized by delayed rises in plasma LH concentration. Since all the control animals in this study were sacrificed during the dioestrous stage of the reproductive cycle, serum LH levels in these animals are comparable. However, the experimental animals were ovariectomized without regard to the stage of the cycle. While it is possible that the present findings are, in part, a consequence of the latter procedure, it seems unlikely that possible effects on LH secretion would have been marked over the 3 weeks duration of the study. Furthermore, the fact that serum LH levels in the intact and sham-operated control animals did not differ significantly, suggests that the operative procedures employed did not contribute to the elevation of serum LH concentration after ovariectomy. Indeed, the mean serum LH concentration in the sham-operated control group (90.5 ± 26.2 ng/ml) was slightly, though not significantly, lower than in the group of intact, dioestrous females (120.4 ± 24.7 ng/ml).

It may be suggested that the absolute serum LH levels in these ovariectomized rats might not be a true reflection of circulating LH concentrations, due to the method of blood collection adopted; this seems unlikely on account of several observations: (a) basal serum LH levels in chronically cannulated ovariectomized rats were not found to

differ significantly from those in ovariectomized rats sacrificed by cervical dislocation (Appendix Table .16.), and (b) studies of basal LH secretion in chronically cannulated, ovariectomized rats (Appendix Table .20.) have revealed mean plasma LH concentrations which were not significantly different from those on days 12, 17 and 21 of the present study.

AUDIOSTIMULATION STUDIES

Evidence in support of the hypothesis that audible sound is capable of influencing reproductive function both in man and in the common laboratory animals has been extensively reviewed in Chapter 1. It is important to emphasize, however, that in the animal studies

a) the various workers employed widely differing sound parameters, thus preventing valid comparison of results;

b) ambient temperature and/or light to dark ratio were frequently left unspecified; and

c) interpretation of results was based largely upon histological examination of the endocrine organs together with alterations in size and weight of these organs following sound treatment.

The postulated effects of audiostimulation on the reproductive system were believed to represent changes in gonadotrophin secretion. This suggestion, however, has been based almost exclusively upon morphological findings, and no investigators have estimated circulating gonadotrophin levels in animals following an audiostimulation programme. Those workers who have reported facilitatory effects of sound treatment upon luteal function (6, 140, 197, 229) have noted ovarian

changes associated with "superovulation" and increased numbers of corpora lutea per ovary. Since luteinizing hormone plays a key role in the induction of ovulation in mammals (59) this study was instituted to measure plasma LH concentrations both before and during each of the 4-day periods of intermittent sound treatment. The choice of "on" (20 secs) "off" (40 secs) sequence and total duration of exposure to the sound (12 hours per day for 3 days) was in keeping with the conditions imposed by previous workers in this laboratory (54, 117). This permitted comparison of the present findings with those of the other workers. Furthermore, in order to obtain an index of mean plasma LH concentration, independent of spontaneous oscillations in plasma LH level, blood was sampled continuously for 1 hour using a peristaltic pump. To avoid blood clotting the animals were heparinized prior to the experiment. Minimal quantities of heparin were used at all times (124), and each animal was examined daily for evidence of alopecia, diarrhoea or bleeding from the mucous membranes (inner surface of lips and gums). None was found.

Studies with intact female rats

A preliminary study using intact female rats indicated that plasma LH concentrations rose during the 3-day audio-stimulation period.

This observation is in agreement with the work of Zondek and Tamari (229) who reported end-organ changes suggestive of enhanced gonadotrophin secretion. Furthermore, Eloff (54) has shown that 80 to 90% of normal female rats develop constant oestrus after 3-4 days of exposure to the sound, and by the 7th day all the sound-stimulated females were in a state of constant oestrus. These end-organ responses suggest very

strongly that they are mediated by a rise in LH output.

Studies with male rats

The reasons for using male rats in this study were as follows:

1) To eliminate the technical problems associated with cyclical variations in plasma LH concentration which would apply in intact female rats;

2) Evidence suggests that both circulating (17) and urinary (14) LH concentrations in normal men are significantly elevated following only 2 days of exposure to an intermittent audiostimulus.

The results of the male audiostimulation study have suggested that none of the frequency-intensity combinations tested exerted any detectable effect upon plasma LH concentration in these animals. (Since all plasma samples were assayed for LH in a single assay, complications due to inter-assay variation have been eliminated from this study.) The findings therefore confirm those of Zondek and Tamari (229) with respect to their failure to detect any morphological changes following audiostimulation in male rats.

Since the sound frequencies and intensities used in this study covered the ranges used by other workers (page 32..) it is unlikely that the results were a consequence of the frequency-intensity combinations used.

It is possible, though unlikely, that a change occurred in plasma LH concentration (a) during the hours of darkness, or (b) soon after commencement of the initial audiostimulation session (i.e. from 8pm to 6am on day 2).

Studies with chronically ovariectomized animals

The experimental findings suggest that the imposed

audiostimulatory conditions exerted no detectable effect upon plasma LH concentrations in chronically ovariectomized animals. However, in the case of the 5KHz/80db study, plasma LH levels on day 4 were significantly higher than on day 1 prior to sound treatment ($P < 0.02$), though neither were significantly different from values in the control study. In addition, the lack of response could not have been a result of depletion of LH from the pituitaries of these rats since plasma LH concentrations increased 3-fold following administration of LRH. A similar 3-fold elevation of plasma LH concentration has been reported by Nakano et al (154) 30 minutes after intravenous injection of lug LRH into bilaterally ovariectomized rats. Since the pituitaries of ovariectomized rats have recently been found to be somewhat less sensitive to exogenous LRH than those of either intact females (126) or oestrogen-progesterone treated ovariectomized rats (126, 154), it now seems logical to suggest that any potential stimulus for LRH (and therefore LH) release might be magnified in the presence of either endogenous or exogenous gonadal steroids. This suggestion is supported by the results of the pilot study into the effects of intermittent audiostimulation on plasma LH levels in intact female rats (FIG 24 and Appendix Table 22).

If the gonadotrophin response to sound is restricted mainly to the intact female rat, as the data suggest, the mechanism of the response may be accounted for either by an oestrogen effect or by the peculiar neural arrangements in the female hypothalamus responsible for the ovulatory release of LH, or a combination of both. Since cyclical (preovulatory) release of LH is mediated via the preoptic area of the hypothalamus (135) and this region of the brain is known to be sensitive to oestrogens (199), it is possible that LH release in

response to sensory input (e.g. reflex ovulation; possible audiostimulatory effects) would also influence gonadotrophin secretion via the anterior hypothalamic area. The latter would be the most likely hypothesis to test, since male rats possess only minimal quantities of oestrogens, while ovariectomized animals lack oestrogens to sensitize the neural system. Furthermore, both groups show a lack of response to audiostimulation, whereas intact females do respond.

LH-SECRETORY RESPONSE TO EXOGENOUS LRH IN CONSCIOUS MALES

In this study the magnitude and duration of the pituitary LH-secretory response to exogenous LRH were measured in male rats. Males, rather than females, were selected in order to eliminate some of the variables associated with altered pituitary sensitivity to LRH which is thought to occur during the oestrous cycle (116).

Most workers have studied variations in the LH-secretory response to LRH in the ovariectomized, oestrogen-progesterone treated animal (37, 79, 126). Relatively few studies have been conducted on male rats. Since numerous workers have investigated the response to exogenous LRH in animals anaesthetized with ether (37, 126), sodium pentobarbitone (18, 138) and urethane (116), and since these anaesthetics have been found to influence the spontaneous LH release in ovariectomized rats (27), these sources of artifact were eliminated by performing the study in conscious, unstressed rats.

The results of this investigation have shown several interesting features regarding plasma LH concentrations following administration of varying doses of LRH:

a) Low doses of LRH (50 and 100ng) evoked maximal LH secretion after 10 minutes, with peak levels 4-5 fold greater than at zero time ($P < 0.001$ and $P < 0.005$ respectively).
While comparable studies have not been observed in available literature, the magnitude of the response is in agreement with the findings of Gay et al (79) and Wedig and Gay (216) following injection of hypothalamic extract (LRH content unspecified) and synthetic LRH (48 and 80ng doses) respectively into ovariectomized rats bearing indwelling carotid cannulae.

b) Higher doses (200, 400 and 800ng) induced peak plasma LH concentrations 20 minutes after injection, with mean plasma LH levels 5-8 fold greater than the pre-injection values ($P < 0.005$ in all cases). Furthermore, the more gradual decline in plasma LH level following a 100ng dose of LRH appears to demonstrate a transition phase between the 10 and 20 minute peak response to the low and high doses of LRH respectively. The fact that higher doses of LRH evoked peak plasma LH concentrations later than did the lower doses, could be explained by either a recirculation effect of LRH on pituitary secretion of LH, or the progressive sensitization of the pituitary gland to LRH (83).

c) Mean plasma LH concentrations 40 minutes after LRH injection were all still significantly elevated above those at zero time.

d) The log dose-response relationship reflects plasma LH concentrations extending between 200ng/ml (with 50ng LRH) and 400ng/ml (with 800ng LRH). Moreover, the linear nature of the curve is in agreement with the original report of Gay et al (78).

With regard to the volume of saline (or saline plus LRH) which was injected into the animals, this was chosen in

accordance with the method used by Libertun et al (126). However, while these workers injected the 0.2ml volume of solution over a period of 60 seconds, in the present study this volume was injected "rapidly" (2-3 seconds) in the form of a bolus. The lack of effect of rapid intravenous injection of saline on mean plasma LH concentration suggests that this method did not significantly influence the LH-secretory response to exogenous LRH. Furthermore, since individual rats are able to respond to several successive injections of hypothalamic extract without any apparent decrease in responsiveness (78), it is suggested that the exposure of the same five rats to each dose of LRH (at 4-day intervals) did not markedly influence these findings, especially since the doses of LRH were administered in a random sequence.

EFFECT OF ANAESTHETICS ON LH RELEASE

Acutely cannulated male rats

Blood collected through aortic cannulae from male rats revealed that, 60 minutes after induction of anaesthesia, LH levels were elevated (after urethane) and depressed (after Sagatal) compared with control unanaesthetized rats sacrificed by cervical dislocation. Exposure of rats to 15 minutes of ether anaesthesia also significantly depressed serum LH concentrations.

The combination of urethane anaesthesia and placement of the cannula in ovariectomized rats was also associated with an elevation of circulating LH concentration and an increase in time interval between LH pulses, as compared with conscious ovariectomized rats.

Since "stress", as induced by etherization and restraint in a rat holder, is capable of increasing LH secretion (52) it was difficult to explain the effects seen in this experiment. The data do not permit differentiation between the effects of surgical trauma (cannulation procedure) on the one hand, and anaesthesia on the other.

Since very little data is available concerning the effects of anaesthesia on circulating LH levels in male rats, it is perhaps appropriate to consider the effects of various anaesthetics on LH levels in females.

The preovulatory release of LH which occurs on the afternoon of pro-oestrus can be blocked by administration of barbiturate (e.g. Nembutal) between noon and 4pm (61, 62). Naftolin et al (151) and Wuttke and Meites (223) have also demonstrated a significant inhibitory effect of pentobarbitone on LH release in adult rats. Some authors have even suggested that ether anaesthesia (as used for venesection in many studies) might be as effective as pentobarbitone in blocking LH secretion (151). In view of the above findings it is reasonable to imagine that the reported inhibitory effect of pentobarbitone and ether on LH release in female rats could be extended to normal male animals. The animals in the present study which were exposed to ether attained anaesthesia of sufficient depth to ensure good muscular relaxation, thereby possibly accounting for the significant depression of serum LH level observed during the use of this anaesthetic.

Since the effect seen with urethane was most dramatic, further studies with this anaesthetic were devised. Urethane is a popular drug employed in neuroendocrine and neuro-physiological research, but considerable controversy exists with

regard to its effect on LH release (28, 95, 128). The information concerning the effect of urethane on basal LH concentrations (128) suggests significant elevation of circulating LH levels one hour after urethane administration to oestrogen-progesterone primed ovariectomized rats. Studies on conscious, unstressed male rats revealed that urethane evoked a transient but significant increase in plasma LH concentrations during the first 30 minutes of anaesthesia. Plasma LH levels declined thereafter to the resting (zero time) values after 1 hour. However, the magnitude of this response was small when compared with that found in the surgically stressed rats. It must therefore be concluded that the LH response in the earlier studies was caused by an anaesthetic component coupled with a surgical stress component.

Finally, the results so far have not indicated the site(s) of action of urethane which, although probably acting centrally, may also have exerted an effect at the pituitary level. In order to test this, the effects of urethane were measured on the LH-secretory response to exogenous LRH in chronically cannulated male rats.

EFFECT OF URETHANE ANAESTHESIA ON THE LH-SECRETORY RESPONSE TO 50ng LRH IN MALE RATS

Urethane anaesthesia appears to have brought about 3 main changes in the LH-secretory response to exogenous LRH, as compared with the response to an equivalent dose of LRH in the conscious animal:

1) 50ng LRH in the urethane-anaesthetized rat evoked a peak response after 40 minutes, with absolute LH levels equivalent to the peak levels induced in the conscious

animal by 400ng LRH;

2) Peak plasma LH concentration under urethane anaesthesia was about 1.75 times greater than the corresponding peak value in the conscious animal; and

3) Plasma LH concentration after 40 minutes was about 3.5 times greater in the urethane-anaesthetized animal.

It thus appears that urethane increases pituitary responsiveness to exogenous LRH and presumably therefore to endogenous LRH. This exaggerated LH-secretory response to 50ng LRH in the urethane-anaesthetized rat could be accounted for by one or more of the following mechanisms.

a) Enhanced secretion of LRH. A rise in plasma LH concentration may well be equated with an increase in LRH output, although there exist other causative factors such as increased sensitivity of the pituitary gonadotrophs to LRH. Since basal LH levels in male rats increased soon after urethane administration, this observation is compatible with the view that urethane anaesthesia might increase endogenous LRH output. Despite the subsequent fall in plasma LH concentrations to baseline values 60 minutes after urethane injection, the earlier enhancement of LRH secretion may have primed the pituitary such that subsequent exogenous LRH administration evoked an exaggerated increase in LH secretion (83).

b) Removal of an inhibitory influence on LH secretion. The data are consistent with the notion that LH secretion is de-inhibited during urethane anaesthesia. Whether this de-inhibition is neurally or chemically mediated will have to be determined by more extensive studies. Under the conditions of the experiment, a very low dose of LRH caused a magnified LH secretory response. This finding, coupled with the fact that unstressed male rats showed increased LH secretion after

urethane administration, suggests an action of urethane at central and/or pituitary site.

With respect to the possible existence of inhibitory influences impinging upon the LH secretory mechanism, recent studies have suggested that melatonin acts by inhibiting luteinizing hormone (LH) secretion (70, 112). Further studies have suggested that aqueous pineal extracts, free of melatonin, possess antigonadotrophic properties in both mice (19, 163) and rats (163).

Since the pineal gland is somewhat removed from the basal hypothalamus, it is difficult, however, to ascribe a function of de-inhibition of LH secretion, via blockage (inhibition) of these substances in the pineal gland, by urethane.

c) Altered metabolism of LH or LRH. The elevation of plasma LH concentration shortly after urethane administration could possibly be accounted for in terms of decreased peripheral degradation of LH or LRH, even in the absence of increased output of either hormone. Studies by Wedig and Gay (216) using pentobarbitone anaesthetized rats, prompted these workers to investigate the possible effects of this anaesthetic on metabolism of circulating LH. These authors concluded that the increase in serum LH levels following anaesthesia revealed an increase in the quantity of LH released.

Preliminary studies in these laboratories have suggested that the half-life of circulating LH is unaffected by urethane anaesthesia. Since very little data is available on the metabolism of LRH in the rat, the possibility that urethane prolongs the time required for degradation of LRH cannot be ignored.

d) Direct effect on the pituitary gland. It is possible that

the more prolonged elevation of plasma LH concentration following LRH injection in the urethane-anaesthetized rat is, at least in part, a consequence of somewhat increased pituitary sensitivity to LRH.

While comparable studies on male rats have not been reported in the literature, Wedig and Gay (216) have demonstrated very similar potentiation of LRH action in oestrogen-progesterone primed ovariectomized rats anaesthetized with pentobarbitone. Blake (27) on the other hand, studied the effects of anaesthetics on pulsatile LH release in the ovariectomized rat. While urethane anaesthesia reversibly inhibited pulsatile LH release, this drug did not inhibit LH release in response to exogenous LRH (100ng) in these rats. In addition, studies with pentobarbitone-anaesthetized ewes (171) suggest that plasma LH levels remained elevated above those in conscious ewes from only 60 minutes after LRH (5µg) injection, while peak plasma LH concentrations were lower in the anaesthetized ewe.

The significant potentiation of LH release by urethane in male rats is in contrast to the findings of Blake and Sawyer (28), Lincoln and Kelly (128) and Haller and Barraclough (95), using female rats, who demonstrated that urethane inhibits the preovulatory rise in circulating LH concentration. Whatever the cause for the different responses, whether it be a sex or strain difference, it has become clear that LH studies performed on anaesthetized animals must be interpreted with caution and cannot readily be extrapolated to the conscious animal.

C O N C L U S I O N S

The results of the various studies show the following:

1) Cervical dislocation of experimental animals leads to artificial elevation of circulating LH concentrations, possibly due to either increased sympathetic discharge, or passive release of LH from the pituitary gland. Thus plasma LH concentrations in chronically cannulated rats provides a more accurate reflection of the true circulating LH levels.

2) Surgical trauma, as occurs in the acutely cannulated animals, might exert a facilitatory effect upon LH secretion. This was particularly apparent in the chronically ovariectomized, urethane-anaesthetized rats since plasma LH levels were significantly greater than those in conscious animals, and pulsatile LH release was, to a certain extent, inhibited. Also, the effects of anaesthetics on LH release in the acutely cannulated male rats, suggested that urethane significantly elevated mean serum LH concentration. Studies with chronically cannulated rats confirmed this effect.

3) The LH-secretory response to 50ng LRH in urethane-anaesthetized rats was markedly different from that in the conscious animals. Since the anaesthetized animals were all chronically cannulated, the pattern of LH secretion in this group could not be ascribed to surgical trauma but rather to the effect of the urethane anaesthesia.

4) The exaggerated LH-secretory response to 50ng LRH in the urethane-anaesthetized rats could be accounted for in terms of either enhanced LRH secretion and/or removal of an inhibitory

influence on LH secretion and/or altered metabolism of LH or LRH and/or a direct action of urethane on the pituitary gland.

A P P E N D I C E S

Appendix TABLE 1....

IODINATION OF RAT LH: SEPHADEX G-50 and CELLULOSE (CF11) ELUTION PATTERNS

SEPHADEX G-50

Tube	Counts/sec
1	27
2	26
3	24
4	22
5	25
6	38
7	16267
8	39122
9	15577
10	5791
11	2843
12	2052
13	1578
14	1631
15	2560
16	24193
17	71473
18	49835
19	15280
20	6527
21	5264
22	3972
23	2722
24	1704
25	1217

CELLULOSE CF 11

Tube	Counts/sec
1	65
2	41
3	2113
4	914
5	154
6	107
7	96
8	92
9	83
10	80
11	77
12	77
The following tubes were eluted with 5% HSA	
13	82
14	80
15	19701
16	23745
17	12347
18	9771
19	9057
20	7638
21	6783

Appendix TABLE 2

FIRST ANTIBODY TITRATION

Tube	Anti-LH dilution (x 10^{-3})	Counts/min		Mean	Mean-NSB	% TC
A S S A Y 1						
TC	-	6747	6331	6539	-	-
NSB	-	1267	1203	1235	-	18.89
1-2	1/4	5277	5124	5201	3966	60.65
3-4	1/8	5355	5009	5182	3947	60.36
5-6	1/16	5108	5105	5107	3872	59.21
7-8	1/32	4924	5024	4974	3739	57.18
9-10	1/64	4419	4324	4372	3137	47.97
A S S A Y 2						
TC	-	9091	9228	9160	-	-
NSB	-	2109	2092	2101	-	22.90
1-2	1/32	6462	6225	6344	4243	46.30
3-4	1/64	5734	5322	5528	3427	37.40
5-6	1/128	4426	4450	4438	2337	25.50
7-8	1/256	3533	3561	3547	1446	15.80

Where TC = Total Counts
NSB = Non-specific binding + background activity
Anti-LH = First antibody

Appendix TABLE 3...

SECOND ANTIBODY TITRATION

Tube	ARGG dilution	Counts/min		Mean	Mean-NSB	% TC
TC		7189	7693	7441		
NSB	1/4	1047	1024	1036		
1		6252	6417	6335	5299	71.2
NSB	1/8	782	765	774		
2		5917	6063	5990	5216	70.1
NSB	1/16	1112	951	1032		
3		3397	2796	3097	2065	27.8
NSB	1/32	1001	1038	1020		
4		1697	1757	1727	707	9.5
NSB	1/64	1140	1005	1073		
5		1126	1115	1121	48	0.7

Where TC = Total Counts
NSB = Non-specific binding + background activity
ARGG = Second antibody

APPENDIX TABLE ....[4]

OPTIMAL FIRST INCUBATION TIME AND ANTI-LH DILUTION

24 HOUR FIRST INCUBATION

Tube	Counts/60 seconds		$\bar{x}$	$\bar{x}$-NSB	% TC ($\bar{x}$-NSB)	B/B_o
S E T A						
Anti-LH Dilution 1/320 x 10^2						
TC	6633	7085	6859			
NSB	1153	1874	1514		22.1	
0%B	6130	6039	6085	4571	66.6	100.0
4ng/ml	6208	6352	6280	4766	69.5	104.4
8	6148	6021	6085	4571	66.6	100
16	6077	5912	5995	4481	65.3	98.0
32	5678	5442	5560	4046	59.0	88.6
62.5	5546	5651	5599	4085	60.0	90.1
125	5041	4992	5017	3503	51.1	76.7
250	3856	3631	3744	2230	32.5	48.8
500	2674	2678	2676	1162	16.9	25.4
S E T B						
Anti-LH Dilution 1/640 x 10^2						
TC	7017	6751	6884			
NSB	1139	1066	1103		16.0	
0%B	5065	5097	5081	3978	57.8	100.0
4ng/ml	5258	5280	5269	4166	60.5	104.7
8	5184	5246	5215	4112	59.7	103.3
16	4873	5007	4940	3837	55.7	96.4
32	4493	4583	4538	3435	49.9	86.3
62.5	3785	3985	3885	2782	40.4	69.9
125	3393	3436	3415	2312	33.6	58.1
250	2589	2569	2579	1476	21.4	37.0
500	1992	1945	1969	866	12.6	21.8

APPENDIX TABLE ...4. (continued)

48 HOUR FIRST INCUBATION

Tube	Counts/60 seconds			$\bar{x}$	$\bar{x}$-NSB	% TC ($\bar{x}$-NSB)	B/Bo
S E T A							
Anti-LH Dilution 1/320 x 10^2							
TC	6972	6883	6928				
NSB	1042	1201	1122			16.2	
0%B	6266	6293	6280	5158		74.5	100.0
4ng/ml	6313	6232	6273	5151		74.4	99.9
8	6100	6240	6170	5048		72.9	97.9
16	5963	6252	6108	4986		72.0	96.6
32	5980	5982	5981	4859		70.1	94.1
62.5	5615	5685	5650	4528		65.4	87.8
125	4954	4765	4860	3738		54.0	72.5
250	3426	3679	3553	2431		35.1	47.1
500	2574	2526	2550	1428		20.6	27.7
S E T B							
Anti-LH Dilution 1/640 x 10^2							
TC	6934	6845	6890				
NSB	1081	1234	1158			16.8	
0%B	5150	4928	5039	3881		56.3	100.0
4ng/ml	5099	5031	5065	3907		56.7	100.7
8	4906	4949	4928	3770		54.7	97.2
16	4588	4724	4656	3498		50.8	90.2
32	4603	4469	4536	3378		49.0	87.0
62.5	3959	3913	3936	2778		40.3	71.6
125	2986	3146	3066	1908		27.7	49.2
250	2172	2295	2234	1076		15.6	27.7
500	1845	1715	1780	622		9.0	16.0

APPENDIX TABLE 4 .... (continued)

72 HOUR FIRST INCUBATION

Tube	Counts/60 seconds			$\bar{x}$	$\bar{x}$-NSB	% TC ($\bar{x}$-NSB)	B/Bo
	S E T A						
	Anti-LH Dilution 1/320 x 10^2						
TC	6792	6975	6884				
NSB	1034	1105	1070			15.5	
0%B	6283	6138	6211	5141		74.7	100.0
4ng/ml	6073	6286	6180	5110		74.2	99.3
8	6009	6006	6008	4938		71.7	96.0
16	6031	6053	6042	4972		72.2	96.7
32	5909	5872	5891	4821		70.0	93.7
62.5	5589	5416	5503	4433		64.4	86.2
125	4599	4602	4601	3531		51.3	68.7
250	3245	3361	3303	2233		32.4	43.4
500	2574	2550	2562	1492		21.7	29.0
	S E T B						
	Anti-LH Dilution 1/640 x 10^2						
TC	7143	7473	7308				
NSB	1016	1048	1032			14.1	
0%B	5320	5412	5366	4334		59.3	100.0
4ng/ml	5266	5328	5297	4265		58.4	98.5
8	5110	4936	5023	3991		54.6	92.1
16	4880	4769	4825	3793		51.9	87.5
32	4427	4355	4391	3359		46.0	77.6
62.5	3687	3904	3796	2764		37.8	63.7
125	2921	2814	2868	1836		25.1	42.3
250	2157	2317	2237	1205		16.5	27.8
500	1845	1859	1852	820		11.2	18.9

APPENDIX TABLE ...5...

SECOND INCUBATION TIME

Tube	Anti-LH Dilution x 10^{-2}	Counts per 6 seconds (Each tube counted for 60 secs)			$\bar{x}$	% TC
72 Hour Second Incubation						
TC		460	(324)	473	467	
NSB		86	89	95	90	19.27
1-3	1:10	128	126	129	128	27.41
4-6	1:80	115	116	114	115	24.63
7-9	1:640	93	97	91	94	20.13
96 Hour Second Incubation						
TC		470	468	463	467	
NSB		82	83	81	82	17.56
1-3	1:10	130	134	131	132	28.27
4-6	1:80	109	106	115	110	23.55
7-9	1:640	90	90	92	91	19.49
120 Hour Second Incubation						
TC		467	(493)	472	470	
NSB		82	87	82	84	17.87
1-3	1:10	130	130	128	130	27.66
4-6	1:80	117	121	114	118	25.11
7-9	1:640	96	93	100	97	20.64
144 Hour Second Incubation						
TC		(468)	491	489	490	
NSB		94	91	95	94	19.18
1-3	1:10	152	150	147	150	30.61
4-6	1:80	140	139	(123)	140	28.57
7-9	1:640	106	108	113	109	22.24

APPENDIX TABLE 6....

CF_{11} ELUTION PATTERNS BEFORE AND AFTER STORAGE OF LABEL

Before storage

Tube	Counts/sec		Tube	Counts/sec	
1	65	Buffer eluate	13	82	5% HSA eluate
2	41		14	80	
3	2,113		15	19,701	
4	914		16	23,745	
5	154		17	12,347	
6	107		18	9,771	
7	96		19	9,057	
8	92		20	7,638	
9	83		21	6,783	
10	80				
11	77				
12	77				

After storage

1	26	Buffer eluate	13	5,333	5% HSA eluate
2	392		14	5,898	
3	45,700		15	4,931	
4	7,339		16	3,993	
5	926		17	3,422	
6	301		18	2,807	
7	180		19	2,407	
8	141		20	2,310	
9	113		21	2,090	
10	98		22	1,825	
11	87		23	1,835	
12	1,187		24	1,683	

APPENDIX TABLE ..7.

STANDARD CURVE USING FIRST CF11 PEAK AS LABEL

Tube	Counts/min		Mean Counts/min
TC	11,243	11,380	11,312
NSB	1,979	1,932	1,956
O%B	1,893	1,956	1,925
4ngLH/ml	1,985	2,014	2,000
8	1,978	1,936	1,957
16	1,880	2,057	1,969
32	2,033	2,022	2,028
62.5	2,035	1,974	2,005
125	1,917	1,972	1,945
250	1,883	1,908	1,896
500	1,946	1,947	1,947

Where TC = Total Counts
NSB = Non-specific binding + background activity
O%B = Zero Binding tube

APPENDIX TABLE 8

DEMONSTRATION OF PARALLELISM BETWEEN STANDARD CURVE AND UNKNOWN SERA FOLLOWING DILUTION

Dilution	B/B_o	ngLH/ml	
"neat"	15.8	560	Serum A
1/2	23.3	290	
1/4	43.8	133	
1/8	66.9	60.9	
1/16	75.5	45.4	
"neat"	18.3	420	Serum B
1/2	31.2	204	
1/4	49.7	108	
1/8	77.3	42.5	
1/16	86.4	25	

Standard Curve

0 ngLH/ml	100
4	91.3
8	86
16	89.9
32	85.8
62.5	64.7
125	48.3
250	25.4
500	16.2
1000	14.6

APPENDIX TABLE ...9.

DEMONSTRATION OF PARALLELISM BETWEEN STANDARD CURVE AND UNKNOWN SERA FOLLOWING DILUTION

Dilution	B/B_o	X	Y	logit Y
Standard curve				
32 ngLH/ml	85.8	1.51	0.858	1.799
62.5	64.7	1.80	0.647	0.606
125	48.3	2.10	0.483	0.068
250	25.4	2.40	0.254	-1.076
500	16.2	2.70	0.162	-1.645
Serum A				
"neat"	15.8		0.158	-1.672
1/2	23.3		0.233	-1.191
1/4	43.8		0.438	-0.250
1/8	66.9		0.669	0.704
1/16	75.5		0.755	1.126
Serum B				
"neat"	18.3		0.183	-1.497
1/2	31.2		0.312	-0.790
1/4	49.7		0.497	-0.012
1/8	77.3		0.773	1.225
1/16	86.4		0.864	1.849

Where, X = log concentration of LH standard

Y = 1 when B/B_o = 100

logit Y = $\log_e (Y/1-Y)$.

See statistical methods page ...98...

APPENDIX TABLE 10

INTER-ASSAY VARIATION - MEANS AND STANDARD DEVIATIONS AT EACH POINT ALONG THE STANDARD CURVE

ngLH/ml	standard curves					Mean B/B_o	SD	CV
	1	2	3	4	5			
4	97.0	91.7	91.3	98.1	98.0	95.2	3.4	3.6
8	93.9	99.1	86.0	96.8	98.9	94.9	5.4	5.7
* 16	92.5	95.2	89.9	100.0	95.4	94.6	3.8	4.0
** 32	87.8	87.8	85.8	91.9	90.3	88.7	2.4	2.7
62.5	75.9	71.6	64.7	74.9	77.0	72.8	5.0	6.9
125	53.8	52.5	48.3	53.5	57.7	53.2	3.4	6.4
250	27.1	26.2	25.4	27.8	33.6	28.0	3.3	11.8
500	17.0	13.8	16.2	18.4	18.8	16.8	2.0	11.9
								6.63%

* $t = 3.2083$
$P < 0.05$

** $t = 10.5582$
$P < 0.001$

Assay No.	Pooled rat serum specimens
1	108ng LH/ml
2	112
3	120
4	109
5	120
6	128
7	122
	117.0 $\bar{x}$
	7.46 SD
	6.38% CV

Where ngLH/ml refers to NIAMD-Rat LH-RP-1
SD = standard deviation
CV = coefficient of variation (SD as percentage of mean)

APPENDIX TABLE 11

INTRA-ASSAY VARIATION - MEAN B/B_o AND STANDARD DEVIATIONS ALONG STEEP PORTION OF THE STANDARD CURVE

ngLH/ml	B/B_o	Mean B/B_o	SD	CV %
4	98.0			
8	98.9			
16	95.4			
32	90.0			
	89.6			
	90.5	90.3	1.8	2.0
	88.1			
	93.1			
62.5	79.4			
	76.1			
	75.4	77.0	1.6	2.1
	77.7			
	76.3			
125	58.9			
	56.4			
	56.9	57.7	1.4	2.4
	59.5			
	57.1			
250	31.4			
	33.6			
	32.1	33.6	2.0	6.0
	36.5			
	34.3			
500	18.8			
				3.1%

APPENDIX TABLE ..12.. METHOD OF STAINING VAGINAL SMEARS

1) Rat vaginal smear taken using toothpick covered with cotton wool, and streaked on to microscope slide previously moistened with a fine film of Physiological saline;

2) Fix in 95% ethanol-diethyl ether (1:1) - 15 mins to 24 hrs

3) Hydrate: 70% - 50% - water

4) Harris' Haematoxylin - 2 mins

5) Rinse in water - 1 min

6) Differentiate in acid alcohol - 3 secs
(1% HCl in 70% alcohol)

7) Rinse in water

8) Blue in 1% lithium carbonate - few seconds

9) Rinse in water

10) Dehydrate: 70% - 95% alcohol

11) 066 - 2 mins

12) 95% alcohol - x 2

13) EA 50 - 3 mins

14) 95% alcohol - x 2

15) Dehydrate: Absolute alcohol

16) Clear: Xylol

17) Mount: DPX

R E S U L T S

Cytoplasm (cornified) - reddish pink
Cytoplasm (non-cornified)- green
Nuclei - blue

APPENDIX TABLE ...[13]

PLASMA LH CONCENTRATIONS IN CONSCIOUS, UNSTRESSED MALE RATS. THE BLOOD SAMPLES WERE DRAWN RANDOMLY FROM 12 ANIMALS AT 8-day intervals.

	(continued)	(continued)
168 ng/ml	25	133
36	67	37
64	26	35
82	70	60
28	39	39
26	39	24
117	36	25
37	107	30
20	37	30
54	24	42
20	44	36
62	30	20
76	45	51
40	102	43
30	44	34
51	72	74
30	47	26
63	43	37
69	23	37
35	22	54
58	51	19
48	28	48
27	52	24
	23	33
	40	

---

n 72

$\bar{x}$ 46.8

SD 27.2

APPENDIX TABLE 14 .... SERUM LH CONCENTRATIONS IN ADULT MALE RATS SACRIFICED BY CERVICAL DISLOCATION

Rat No.	Serum LH (ng/ml)	
1	87	
2	89	
3	68	
4	195	
5	93	
6	108	
7	99	
8	96	
9	105	
	9	n
	104.4	$\bar{x}$
	35.9	SD

APPENDIX TABLE 15 .... SERUM LH CONCENTRATIONS IN BOTH CONSCIOUS AND CERVICALLY DISLOCATED FEMALE RATS DURING THE DIOESTROUS AND METOESTROUS (RESTING) PHASES OF THE REPRODUCTIVE CYCLE.

Conscious		Cervical Dislocation
70 ng/ml		113 ng/ml
85		95
133		64
57		118
72		63
52		97
89		125
91		153
85		107
49		
88		
64		
12	n	9
77.9	$\bar{x}$	103.9
22.9	SD	28.6

APPENDIX TABLE ....[16] PLASMA LH CONCENTRATIONS IN BOTH CONSCIOUS AND CERVICALLY DISLOCATED LONG-TERM BILATERALLY OVARIECTOMIZED RATS

Conscious		Cervical Dislocation
635 ng/ml		590 ng/ml
683		820
693		760
750		640
640		925
720		815
870		560
665		485
665		725
625		640
670		610
780		810
760		715
710		700
660		
15	n	14
701.7	$\bar{x}$	699.6
65.8	SD	120.3

APPENDIX TABLE ...[17] APPEARANCE OF VAGINAL SMEARS ON EACH DAY OF 2 CONSECUTIVE OESTROUS CYCLES

Day	Rat 1	Rat 2	Rat 3
1	Ker +++ Epith.cells *	Ker Leuc	Ker +(+) Small Nucl. cells +++ *
2	Ker ++(+) Nucl.cells +(+)**	Ker ++(+) Nucl.cells	Ker +++ Nucl.cells + **
3	Ker +(+) Nucl.cells ++	Ker +++ Nucl.cells + **	Ker + Nucl.cells ++ Leuc ++(+)
4	Ker ++ Nucl.cells + Leuc ++	Ker +++ Epith.cells Leuc	Ker + Nucl.cells ++ Leuc +(+)
5	Ker +++ Nucl.cells + *	Ker ++ Nucl.cells ++ Leuc +	Ker +++ Nucl.cells + *
6	Ker +++ Nucl.cells + **	Ker +(+) Small Nucl. cells +(+) *	Ker +++ Nucl.cells **
7	Ker +(+) Nucl.cells Leuc +++	Ker +++ Nucl.cells ++ **	Ker +++ Nucl.cells ++ Leuc ?
8	Ker + Nucl.cells + Leuc +++	Ker ++ Nucl.cells +(+) Leuc	Ker Nucl.cells ++ Leuc +(+)

Where:
Ker = keratin
Nucl.cells = nucleated cells (epithelial)
Epith.cells = epithelial cells (nuclei not prominent)
Leuc = leucocytes
* = pro-oestrus
** = oestrus

APPENDIX TABLE ....[18] FLUCTUATIONS IN SERUM LH CONCENTRATIONS DURING THE NORMAL RAT OESTROUS CYCLE

Stage	Rat No.	NIAMD-Rat LH-RP-1 (ng/ml) Plasma LH	Mean LH	SD	
Dioestrous	1	70.2 ; 84.9			
	2	133 ; 57.4	78.2	29.3	
	3	72 ; 51.8			
Pro-oestrous	1	775 ; 410			
	2	204 ; 194	345.3	224.8	*
	3	264 ; 225			
Oestrous	1	320 ; 274			
	2	177 ; 375	256.7	79.8	**
	3	200 ; 194			
Metoestrous	1	88.5 ; 91			
	2	84.8 ; 48.5	77.5	17.3	
	3	88 ; 64			

** = $P < 0.001$
* = $P < 0.025$

Compared with dioestrous values

APPENDIX . TABLE ...19. EFFECT OF BILATERAL OVARIECTOMY ON RAT SERUM LH CONCENTRATION

Experiment	Rat No.	Serum LH (ng/ml)		
Sham-operated controls	1	113		
	2	94.5		
	3	64		
	4	118		
	5	62.8		
		90.5	Mean	
		26.2	SD	
Intact control females	1	96.5		
	2	125		
	3	153		
	4	107		
		120.4	Mean	
		24.7	SD	
7 Days after operation	1	460		
	2	740		
	3	450		
	4	303		
	5	535		
		497.6	Mean	*
		159.5	SD	
12 Days after operation	1	590		
	2	820		
	3	760		
	4	640		
		702.5	Mean	**
		106	SD	
17 Days after operation	1	925		
	2	815		
	3	560		
	4	485		
	5	725		
		702	Mean	**
		180.5	SD	
21 Days after operation	1	640		
	2	610		
	3	810		
	4	715		
	5	700		
		695	Mean	**
		77.3	SD	

** = $P < 0.001$
* = $P < 0.005$
as compared with sham-operated control animals.

APPENDIX TABLE ...20 PATTERN OF BASAL LH SECRETION IN FREE-RUNNING OVARIECTOMIZED RATS

	Plasma LH (ng/ml)		
Minutes	Rat I	Rat II	Rat III
5	775	760	800
10	595	800	700
15	515	660	700
20	540	725	730
25	775	775	610
30	625	720	680
35	500	590	605
40	660	515	710
45	800	500	710
50	605	785	540
55	740	700	475
60	670	605	785
65	540	760	915
70	555	660	740
Mean	635.4	682.5	692.9
SD	103.4	98.2	110.7

"t" values:

Between I and II - 1.19096 ($P < 0.3$)
I and III - 1.36871 ($P < 0.2$)
II and III - 0.25341 ($P < 0.9$)

APPENDIX TABLE ....[21] PLASMA LH CONCENTRATIONS IN OVARIECTOMIZED RATS BEFORE (Day 1) AND AFTER (Day 4) 3 SESSIONS OF SOUND TREATMENT

Sound settings	Rats	Day 1	Day 4	$\bar{d}$	Sd	"t" (1 vs 4)
0 KHz/0db (control)	I	750	750			
	II	640	760	40	69.3	0.9999
	III	720	720			P< 0.5
	Mean LH	703.3	743.3ng/ml			
	SD	56.9	20.8			
10 KHz/80db	I	870	760			
	II	665	670	3.33	102.8	0.0562
	III	665	760			P< .975
	Mean LH	733.3	730 ng/ml			
	SD	118.4	52			
*	"t"	0.3230	0.3358			
	P<	0.8	0.8			
5 KHz/80db	I	625	670			
	II	670	735	56.7	10.4	9.4299
	III	780	840			P< 0.02
	Mean LH	691.7	748.3 ng/ml			
	SD	79.7	85.8			
*	"t"	0.1675	0.0801			
	P<	0.9	0.95			
2.5 KHz/80db	I	760	750			
	II	710	750	83.3	121	1.1932
	III	660	880			P< 0.4
	Mean LH	710	793.3 ng/ml			
	SD	50	75.1			
*	"t"	0.1251	0.9074			
	P<	0.95	0.5			

Where $\bar{d}$ = mean difference

SD = standard deviation of the differences

"t"(1 vs 4) = t value between LH values on days 1 & 4

* "t" = t value between LH values in the control and sound treated groups on a given day.

APPENDIX TABLE ....[22]

EFFECT OF 10 KHz/65db INTERMITTENT AUDIOSTIMULATION ON PLASMA LH CONCENTRATIONS IN INTACT FEMALE RATS

	Day	Rats		Mean LH (ng/ml)
		1	2	
Control	1	133	182	157.5
Sound	2	136	195	165.5
Sound	3	170	243	206.5
Sound	4	217	220	218.5

APPENDIX TABLE ...23 PLASMA LH CONCENTRATIONS IN CONSCIOUS MALE RATS FOLLOWING RAPID INTRAVENOUS INJECTION OF LRH

		(Minutes after LRH)					
	Rat	0	5	10	20	40	80
0ng LRH	I	47	70	68	40	< 30	66
(saline control)	II	52	40	< 30(12)	60	< 30	<30(5)
	$\bar{x}$	49.5	55	40	50	< 30	35.5ng/ml
50ng LRH	I	80	198	250	174	102	72
	II	46	130	172	192	117	66
	III	72	121	204	149	106	50
	IV	71	218	240	179	100	43
	V	45	127	146	139	66	54
	$\bar{x}$	62.8	158.8	202.4*	166.6	98.2	57 ng/ml
	SD	16.2	45.6	44.1	21.9	19.2	11.8
			d	e	e	b	
100ng LRH	I	38	167	248	192	90	65
	II	48	136	154	191	90	40
	III	63	155	208	164	115	50
	IV	63	306	424	452	164	81
	V	43	139	196	184	96	52
	$\bar{x}$	51	180.6	246*	236.6	111	57.6ng/ml
	SD	11.5	71.2	105	120.9	31.4	15.8
			b	b	a	d	
200ng LRH	I	77	234	376	384	210	102
	II	28	116	185	236	146	105
	III	70	131	164	194	118	67
	IV	49	216	336	398	248	91
	V	41	139	195	208	130	75
	$\bar{x}$	53	167.2	251.2	284*	170.4	88 ng/ml
	SD	20.3	53.8	97.4	99	56	16.6
			c	c	c	c	

APPENDIX TABLE ...23 (continued)

		(Minutes after LRH)					
	Rat	0	5	10	20	40	80
400ng LRH	I	48	282	388	496	308	86
	II	55	124	204	268	189	71
	III	47	107	193	256	121	45
	IV	41	236	464	566	368	86
	V	32	114	156	192	125	41
	$\bar{x}$	44.6	172.6	281	355.6*	222.2	65.8ng/ml
	SD	8.6	80.8	136.2	164.6	111.1	21.7
			a	b	b	a	
800ng LRH	I	57	242	374	480	378	97
	II	65	266	286	316	234	102
	III	54	103	218	284	182	86
	IV	48	244	480	610	482	133
	V	36	126	200	276	324	125
	$\bar{x}$	52	196.2	311.6	393.2*	320	108.6ng/ml
	SD	10.8	75.6	116.3	146.8	118.4	19.7
			c	c	c	c	b

Mean	52.7	of "0 min" values
SD	14.2	

* Peak plasma LH concentrations.

a, b, c, d & e represent P values of less than 0.025, 0.02, 0.01, 0.005 and 0.001 respectively compared with 0-minute LH concentrations using paired Student's t-test.

APPENDIX TABLE [23] .... (continued)

PEAK PLASMA LH CONCENTRATIONS ATTAINED WITH EACH DOSE OF LRH

	RAT NOS.						
	I	II	III	IV	V	$\bar{x}$	SD
50ng LRH	250	192	204	240	146	206.4	41.5
100	248	191	208	452	196	259.0	110.2
200	384	236	194	398	208	284.0	99.0
400	496	268	256	566	192	355.6	164.6
800	480	316	284	610	324	402.8	138.5

APPENDIX TABLE 24....

PLASMA LH CONCENTRATIONS IN OVARIECTOMIZED RATS BEFORE AND AFTER RAPID INTRAVENOUS INJECTION OF 800ng LRH

	Before LRH	After LRH	
Rat I	750	2,190	
II	760	2,360	
III	720	2,250	
Mean LH	743.3	2,266.7	ng/ml
SD	20.8	86.2	

t value = 24.2960

$P < 0.001$

APPENDIX TABLE 25 .... EFFECTS OF THE ANAESTHETICS, URETHANE, INACTIN, SAGATAL AND ETHER ON RAT SERUM LH CONCENTRATION (ng/ml)

	(Mins after administration of anaesthetic)						
	30	40	50	60	70	80	90
URETHANE							
Rat 1	167	152	137	130	152	164	161
2	338	298	197				
3		197	169	169	120	139	139
4	198	165	139	118	162		
5	153	158	157	169	167		
Mean LH	214	194	159.8	146.5	150.3	151.5	150
SD	84.8	60.7	24.6	26.4	21.1	17.7	15.6
INACTIN							
Rat 1	105	80	64	68			
2	112	72	68	62	55	57	50
3	72	64	53	49	45	44	51
4	130	111	86	88	76	69	58
5	68	60	56	53	77	77	
Mean LH	97.4	77.4	67.4	64	63.3	61.8	53
SD	26.7	20.3	11.9	15.3	15.8	14.4	4.4
SAGATAL							
Rat 1	78	64	61	68	68	63	55
2			31	37	33	45	61
3		65	66	55	55	58	65
4		85	70		74	70	67
5			49		50		70
6		68	67	87			
Mean LH	78	70.5	57.3	61.8	56	59	63.6
SD	-	9.8	14.9	21.1	16.1	10.6	5.8

Males exposed to 15 mins of ether anaesthesia before sacrifice

Rat No.	Serum LH (ng/ml)	
1	61	
2	68.5	
3	47	
4	36.8	
	53.3	Mean LH
	14.2	SD

APPENDIX TABLE 25 .... (continued)

Male rats sacrificed by cervical dislocation:

Rat No.	Serum LH (ng/ml)	
1	86.5	
2	88.5	
3	68	
4	195	
5	92.5	
6	108	
7	98.5	
8	96	
9	105	
	104.2	Mean LH
	36	SD

APPENDIX TABLE ....[26] PATTERN OF BASAL LH SECRETION IN OVARIECTOMIZED RATS DURING URETHANE ANAESTHESIA

Minutes	Plasma LH (ng/ml) Rat X	Rat Y
5	845	720
10	900	1055
15	1010	1345
20	1075	1345
25	1010	1165
30	1095	1035
35	1110	1110
40	1045	855
45	1035	720
50	900	760
55	740	660
60	920	740
65	780	575
70	900	665
75	855	990
Mean	948	916
SD	115.6	251.8

"t" value = 0.43216

($P < 0.7$)

APPENDIX TABLE ...27.

STUDENT'S t-test BETWEEN MEAN PLASMA LH CONCENTRATIONS IN FREE-RUNNING OVARIECTOMIZED RATS I, II & III AND URETHANE-ANAESTHETIZED RATS X & Y

Free-running rats I, II & III		Urethane-anaesthetized rats X & Y
42	(n)	30
670.2	Mean LH (ng/ml)	932
104.7	SD	193.2

t value = 7.2901

$P < 0.001$

APPENDIX TABLE ....[28] EFFECT OF URETHANE ON LH-SECRETORY RESPONSE TO 50ng LRH IN MALE RATS. URETHANE (OR SALINE) INJECTED AT -60 MINUTES

		Minutes after LRH or saline					
Urethane + 50ng LRH		0	10	40	80	120	
Rat	1	79	205	212	-	-	
	2	102	375	500+ *	-	-	
	3	85	182	323	-	-	
	4	24	202	465	380	-	
	5	77	490	500	158	113	
	6	57	233	250	98	50	
Mean LH		70.7	281.2	350	212	81.5	ng/ml
SD		27.1	123.8	128	148.6		
Urethane + saline							
Rat	A	42	60	41	39	36	
	B	26	21	31	26	29	
	C	82	82	86	59	40	
Mean LH		50	54.3	52.7	41.3	35	ng/ml
SD		28.8	30.9	29.3	16.6	5.6	
Intraperitoneal saline + 50ng LRH							
Rat	1	65	197	118	-	-	
	2	60	232	113	-	-	
Mean LH		62.5	214.5	115.5	-	-	ng/ml

* No absolute value available. This figure was omitted for the statistical analyses.

B I B L I O G R A P H Y

B I B L I O G R A P H Y

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